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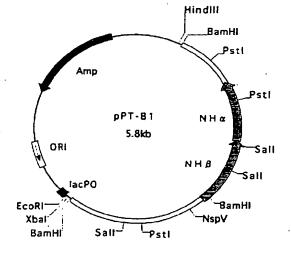
The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) Nitrile hydratase, derivatives thereof and methods of producing compounds

(57) The present invention provides the amino acid sequence and base sequence of a <u>Pseudonocardia</u> thermophila-derived nitrile hydratase, provides further a method for changing its amino acid sequence and base sequence without substantially changing the functions of said nitrile hydratase, and nitrile hydratases having a base sequence and an amino acid sequence as

changed on the basis of said method, and provides furthermore a recombinant plasmid having the gene of said nitrile hydratase, a transformant containing said recombinant plasmid, a method of using said transformant for producing said enzyme, and a method of using said transformant for producing the corresponding amide compound from a nitrile compound.

Fig-1



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Description

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The present invention relates to a novel amino acid sequence that comprises the α -subunit and the β -subunit of a nitrile hydratase as derived from <u>Pseudonocardia thermophila</u> JCM3095 (hereinafter referred to as <u>Pseudonocardia thermophila</u>), and to a novel gene sequence that codes for the α -subunit and the β -subunit of said enzyme. Further, it relates to a recombinant plasmid containing said gene, to a transformant strain as transformed with said recombinant plasmid, to a method for producing a nitrile hydratase using said transformant strain, and to a method for processing a nitrile compound with a culture as obtained by cultivating cells of said transformant strain, with the cultivated cells or with a product as obtained by processing the cultivated cells, to produce the corresponding amide compound from said nitrile compound.

Regarding the technique of hydrating the nitrile group of a nitrile compound to convert it into an amido group, thereby producing the corresponding amide compound from said nitrile compound, there have heretofore been known chemical methods of heating a nitrile compound in the presence of an acid or alkali or in the presence of a copper catalyst.

On the other hand, recently, an enzyme, nitrile hydratase that has a nitrile-hydrating activity of hydrating a nitrile group to convert it into an amido group has been found, and methods of processing a nitrile compound with said enzyme or cells of microorganisms that contain said enzyme to produce the corresponding amide compound from said nitrile compound have already been disclosed (see Japanese Patent Publication Nos. 56-17918, 62-21519, 62-31914, 59-37951, 4-4873 and 6-55148). It is known that these methods are more advantageous than the conventional chemical methods in that the conversion and the selectivity from nitrile compounds into the corresponding amide compounds are higher in the former than those in the latter.

To produce amide compounds from nitrile compounds with such a nitrile hydratase on an industrial scale, it is desirable to lower the production costs in producing in said enzyme and in producing amide compounds using said enzyme. More concretely, for this, it is necessary to elevate the production rate for producing an amide compound per a unit cell weight and per a unit time (hereinafter referred to as cell activity). Given the situation, various attempts have been being made to clone said enzyme, nitrile hydratase for the purpose of expressing a large amount of said enzyme through genetic engineering means of using the gene of said enzyme. For example, known are the means of using cells of Corynebacterium (see Japanese Patent Application Laid-Open No. 3-251184), cells of Rhodococcus rhodoclous (see Japanese Patent Application Laid-Open No. 4-211379), cells of Rhizobium (see Japanese Patent Application Laid-Open No. 6-25296, or cells of Klebsiella (see Japanese Patent Application Laid-Open No. 6-303971). However, for Corynebacterium and Rhodococcus rhodoclous, it is reported that the nitrile hydratase activity of the transformants of Escherichia coli with any of these is extremely weak (see TIBTECH Vol. 10, pp. 402-408, 1992). Thus, it was not always possible to obtain the intended transformants with high cell activity through such genetic engineering means.

We, the present inventors have succeeded in, for the first time, isolating a nitrile hydratase gene from cells of Pseudonocardia thermophila JCM3095, which we found having a nitrile hydratase activity, and in clarifying its amino acid sequence and gene sequence, and have also succeeded in the formation of genetic recombinant cells capable of expressing a large amount of said gene. On the basis of these findings, we have completed the present invention.

Specifically, the present invention seeks to provide the amino acid sequence and the gene sequence of a <u>Pseudonocardia thermophila</u>-derived nitrile hydratase. The present invention further seeks to provide a recombinant plasmid having said gene, a transformant having said plasmid, a method for producing said enzyme using cells of said transformant, and a method of processing a nitrile compound with cells of said transformant to produce the corresponding amide compound from it.

Specific embodiments of the invention will now be described with reference to the accompanying drawings, in which:

Fig. 1 shows a restriction endonuclease cleavage map of a plasmid pPT-B1.

Fig. 2 shows a restriction endonuclease cleavage map of a plasmid pPT-DB1.

In these:

Amp indicates a gene coding for β-lactamase,

ORI indicates the replication-starting site in a CoIE1 system,

lacPO indicates the promoter and operator region in pUC18-derived lactose operon,

NH α indicates a gene coding for the α -subunit of <u>Pseudonocardia</u> thermophila-derived nitrile hydratase,

NHβ indicates a gene coding for the β-subunit of Pseudonocardia thermophila-derived nitrile hydratase, and

(Xbal/NspV) indicates the site for self-ligation between Xbal and NspV as attained after blunting.

Now, the present invention is described hereinunder. The nitrile hydratase gene of the present invention basically comprises the amino acid sequences of Sequence Number 1 and Sequence Number 2 in Sequence Listing. However,

even in transcription and translation from a template of a gene having the same base sequence, one or more amino acids near the N-terminal of an amino acid sequence may be deleted or one or more amino acids may be added to the N-terminal thereof to give mutants having the same enzymatic activity as that of the original, depending on the type of hosts to which the gene is introduced, the components constituting the nutrient medium to be used for incubation of the hosts as well as the composition of the medium, and the temperature and the pH of the medium being used for the incubation, and owing to the modification of the intracellular enzyme after its production through the genetic expression. In addition, with the recent progress in recombinant DNA technology, it has become possible relatively with ease to modify an enzyme, without substantially changing its activity, through substitution, deletion or addition of one or more amino acids in the amino acid sequence of the enzyme. Moreover, recently, various attempts are being made to produce mutant enzymes with improved industrial values, for example, having improved organic solvent resistance or having varied substrate specificity, through substitution, deletion or addition of one or more amino acids in amino acid sequences of enzymes. In view of such technical level in the art, the nitrile hydratase as referred to herein shall include not only those having the amino acid sequences of Sequence Numbers 1 and 2 in Sequence Listing but also any other mutants having amino acid sequences as modified through substitution, deletion or addition of one or more amino acids in said amino acid sequences, provided that they have the intended nitrile hydratase activity, and the present invention shall encompass any and every nitrile hydratase having any of such amino acid sequences and such modified amino acid sequences.

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Specifically, the present invention is directed to the nitrile hydratase comprising the α -subunit having the amino acid sequence of 205 amino acids of Sequence Number 1 in Sequence Listing and the β -subunit having the amino acid sequence of 233 amino acids of Sequence Number 2 in Sequence Listing. Needless-to-say, the present invention encompasses any and every nitrile hydratase comprising, as the constitutive element, either one or both of modified α -subunit and β -subunit to be constructed through partial substitution, deletion or addition of one or more amino acids in the amino acid sequences of Sequence Numbers 1 and 2 in Sequence Listing, and having a nitrile-hydrating activity.

In the present invention, the base sequence coding for the α -subunit to be expressed by the amino acid sequence of 205 amino acids of Sequence Number 1 in Sequence Listing is within the scope of the gene coding for the α -subunit constituting the nitrile hydratase of the invention. In addition, provided that any and every protein comprising, as the constitutive element, any modified α -subunit to be constructed through partial substitution, deletion or addition of one or more amino acids in the amino acid sequence of Sequence Number 1 in Sequence Listing, have a nitrile-hydrating activity, any and every base sequence coding for such a modified α -subunit is within the scope of the nitrile hydratase gene of the present invention. Similarly, in the present invention, the base sequence coding for the β -subunit to be expressed by the amino acid sequence of 233 amino acids of Sequence Number 2 in Sequence Listing is within the scope of the gene coding for the β -subunit constituting the nitrile hydratase of the invention. Also similarly, in addition, provided that any and every protein comprising, as the constitutive element, any modified β -subunit to be constructed through partial substitution, deletion or addition of one or more amino acids in the amino acid sequence of Sequence Number 2 in Sequence Listing, have a nitrile-hydratase gene of the present invention.

The nitrile hydratase gene of the present invention basically comprises the base sequences of Sequence Numbers 3 and 4 in Sequence Listing. With the recent progress in recombinant DNA technology, however, it has become possible relatively with ease to substitute the base sequence of a DNA to be the template in genetic translation to produce an enzyme, with any other base sequence, without substantially changing the amino acid sequence of said enzyme. In addition, it has become also possible to modify the amino acid sequence of an enzyme into modified ones with substitution, deletion or addition of one or more constitutive amino acids therein, without substantially changing the enzymatic activity of the enzyme, through substitution, deletion or addition in the base sequence of the DNA to be the template in genetic translation to produce the intended enzyme. In view of such technical level in the art, the nitrile hydratase gene as referred to herein shall include not only those having the DNA base sequences of Sequence Numbers 3 and 4 in Sequence Listing but also any other mutants having DNA base sequences as modified through substitution, deletion or addition of one or more bases in said DNA base sequences, provided that they can function as the template for proteins having a nitrile hydratase activity. Specifically, the present invention is directed to the gene coding for a nitrile hydratase, in which the α-subunit comprises the base sequence of 618 bases of Sequence Number 3 in Sequence Listing, while the β-subunit comprises the base sequences of 702 bases of Sequence Number 4 in Sequence Listing. In the present invention, in addition, provided that any and every protein comprising, as the constitutive element, either one or both of the α -subunit of an amino acid sequence which is encoded by any modified base sequence as constructed through partial substitution, deletion or addition in the base sequence of Sequence Number 3 in Sequence Listing, and the β-subunit of an amino acid sequence which is encoded by any modified base sequence as constructed through partial substitution, deletion or addition in the base sequence of Sequence Number 4 in Sequence Listing, have a nitrile-hydrating activity, any and every gene coding for either one or both of such modified αsubunit and β-subunit is within the scope of the nitrile hydratase gene of the present invention.

The present invention further includes the construction of a recombinant plasmid having the nitrile hydratase gene

of the invention as inserted thereinto, and the transformation of any desired microorganisms with said recombinant plasmid to give transformants. Moreover, the present invention still includes the production of the intended enzyme through incubation of the resulting transformant cells in ordinary nutrient media, and even the production of amide compounds through contact of said transformant cells, which produce the intended enzyme, with nitrile compounds in aqueous media to convert said nitrile compounds into the corresponding amide compounds.

The recombinant plasmid of the present invention is a plasmid as constructed by inserting the nitrile hydratase gene into a plasmid vector having therein a control region necessary for the expression of said gene and a region necessary for the self-replication thereof, and this can be introduced into any desired host to make it produce the enzyme, nitrile hydratase. As the host employable herein, mentioned is Escherichia coli, as in the following examples, which, however, is not limitative. Apart from this, any other microorganisms of the genus Bacillus (such as Bacillus subtilis), yeasts, actinomyces and others are also employable. The control region necessary for the expression comprises a promoter sequence (including the operator sequence for control of transcription), a ribosome-binding sequence (SD sequence), and a transcription-terminating sequence. Concretely, the promoter sequence includes a trp promoter of tryptophan operon and a lac promoter of lactose operon that are derived from Escherichia coli, a PL promoter and a PR promoter that are derived from lambda phage, and a glucuronic acid synthetase promoter (gnt), an alkali protease promoter (apr), a neutral protease promoter (npr) and an -amylase promoter (amy) that are derived from Bacillus subtilis. In addition to these, also employable herein are a tac promoter and the like that have been modified or designed for themselves. The ribosome-binding sequence includes, for example, those derived from Escherichia coli and Bacillus subtilis, as well as the sequence intrinsic to Pseudonocardia thermophila as in the present invention. Any of these is employable in the present invention with no specific limitation, provided that it functions in desired hosts such as Escherichia coli and Bacillus subtilis. For example, a consensus sequence comprising a series of 4 or more continuous bases that are complementary to the 3'-terminal region of 16S ribosome RNA may be prepared through DNA synthesis and used herein as the ribosome-binding sequence. The transcription-terminating sequence is not always necessary, for which, however, employable is one not depending on any ρ-factor, for example, a lipoprotein terminator or a trp operon terminator. Regarding the sequence of the control region on the recombinant plasmid, it is desirable that the promoter sequence, the ribosome-binding sequence, the nitrile hydratase gene, and the transcription-terminating sequence are in that order, starting from the upstream side of the 5'-terminal end of the region. In the control region of that type, the α -subunit gene and the β -subunit gene may be expressed for the respective independent cistrons or, alternatively, the two may be expressed together in the polycistronic manner using same control region common to the

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As examples of the plasmid vector that satisfies the above-mentioned requirements, mentioned are pBR322, pUC18, Bluescript II SK(+), pKK223-3 and pSC101 all having a region self-replicable in <u>Escherichia coli</u>; and pUB110, pTZ4, pC194, p11, \$\phi\$1 and \$\phi\$105 all having a region self-replicable in <u>Bacillus subtilis</u>. As examples of the plasmid vector that is self-replicable in two or more different hosts, mentioned are pHV14, TRp7, YEp7 and pBS7.

For the method of inserting the gene that codes for the nitrile hydratase of the invention into the plasmid vector that has the region necessary for the expression of said gene to construct the intended recombinant plasmid of the invention, for the method of transforming a desired host with said recombinant plasmid, and for the method of producing the intended nitrile hydratase of the invention in the cells of the resulting transformant, employable are any ordinary methods and hosts that are generally known in the field of molecular biology, bioengineering and genetic engineering, such as those described in "Molecular Cloning. 2nd Edition" (T. Maniatis et al.; Cold Spring Harbor Laboratory Press, 1988). As the media for incubating transformant cells, generally used are LB media and M9 media. More preferably, these media for use in the present invention comprise Fe ions and Co ions in an amount 0.1 µg/ml or more.

To produce the corresponding amide compound from a nitrile compound by the use of the nitrile hydratase or the transformant cells of the present invention as prepared in the manner mentioned above, a desired nitrile compound is kept in contact with the culture of the transformant cells, or with the transformant cells themselves as isolated from the culture thereof, or with a product as produced by processing the transformant cells, or with a nitrile hydratase as isolated and purified from the transformant cells, in an aqueous medium. The temperature for the contact is not specifically defined, but is preferably within the range within which the nitrile hydratase is not deactivated, more preferably within the range between 0°C and 50°C. The nitrile compound to be processed is not specifically defined, provided that it may be a substrate on which the nitrile hydratase of the present invention can acts. Preferably, however, it includes, for example, nitrile compounds having from 2 to 4 carbon atoms, such as typically acetonitrile, propionitrile, acrylonitrile, methacrylonitrile, n-butyronitrile, isobutyronitrile, crotononitrile and a-hydroxyisobutyronitrile. The concentration of the nitrile compound in the aqueous medium is not specifically defined at all, provided that it is within the range that does not exceed the degree of maximum solubility of the nitrile compound in said medium. Preferably, however, the nitrile concentration may be 5 % by weight or lower, more preferably 2 % by weight or lower, in consideration of the activity of the enzyme that may not be deactivated by the nitrile compound.

A series of steps are summarized hereinunder, which we, the present inventors have employed before the clarification of the amino acid sequences and the base sequences of the Pseudonocardia thermophila-derived nitrile hy-

dratase of Sequences Numbers 1 to 4 in Sequence Listing. <u>Pseudonocardia thermophila</u> JCM3095 has been being stored in Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN) of 2-I, Hirosawa, Wako-shi, Saitama-ken, Japan under a code number of JCM3095, which is now available to anyone who requests it.

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- (1) Cells of the strain are incubated, isolated, disrupted, and subjected to ammonium sulfate fractionation, anion-exchange chromatography, gel filtration chromatography and hydrophobic chromatography to thereby isolate and purify the nitrile hydratase therefrom, of which the amino acid sequences of 7 residues in the N-terminal of the α -subunit and the β -subunit were sequenced.
- (2) Based on the thus-sequenced N-terminal amino acid sequences, oligonucleotide primers for genetic amplification were prepared. A chromosome DNA was prepared from the cells. Through PCR using these primers and the chromosome DNA as the template, obtained were amplified DNA products.
- (3) The chromosome DNA was partially cleaved with restriction endonucleases to collect DNA fragments of from about 1500 bp to about 4000 bp. Each DNA fragment was linked to a plasmid vector, with which <u>Escherichia coli</u> was transformed to give a plasmid library.
- (4) Through colony hybridization using, as the probe, the amplified DNA products as obtained in the previous (2), positive clones comprising the DNA fragment that codes for the nitrile hydratase were selected from the plasmid library.
- (5) From the positive clones, extracted was the plasmid DNA, and the whole base sequence of the insert fragments was sequenced, whereby the base sequence of the gene that codes for the α -subunit and the β -subunit of the nitrile hydratase was identified. Comparing the amino acid sequences of the α -subunit and the β -subunit that may be presumed from the thus-sequenced base sequence with the N-terminal amino sequences of 7 residues of the both subunits as obtained in the previous (1), it was verified that the base sequence as sequenced previously codes for the nitrile hydratase.
- (6) The DNA fragment containing the nitrile hydratase gene was re-prepared from the plasmid of the positive clone, as obtained in the previous (4) and inserted into a plasmid vector having a suitable promoter.
- (7) The plasmid as obtained in the pervious (6) was introduced into suitable host cells to obtain transformant cells. These transformant cells were incubated, and isolated from the culture. These cells were then kept into contact with acrylonitrile in an aqueous medium, whereupon the formation of acrylamide was confirmed.

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EXAMPLES

Now, the present invention is described in more detail with reference to the following examples, which, however, are not intended to restrict the scope of the invention.

Example 1:

Purification of Nitrile Hydratase from Pseudonocardia thermophila JCM3095:

Cells of Pseudonocardia thermophila JCM3095 were incubated in a nutrient broth medium (pH 7.5) containing 0.2 % of methacrylamide, at 50°C for 3 days. Through centrifugation of the culture (8000 x G, 30 minutes) obtained were 3 g of wet cells. These were suspended in 7 g of KPB (0.1 M potassium phosphate buffer; pH 7.0), and disrupted using an ultrasonic cell disruptor to give a liquid comprising cell debris. 2.7 g of ammonium sulfate was added to the liquid comprising cell debris, which was gently stirred at 4°C for 1 hour and then centrifuged (25000 x G, 30 minutes) to remove the insoluble solids therefrom. 1.2 g of ammonium sulfate was added to 90 g of the resulting supernatant, which was gently stirred at 4°C for 1 hour and then centrifuged (25000 x G, 30 minutes) to collect the precipitate. The precipitate was dissolved in 1 ml of KPB, and dialyzed against 2 liters of the same KPB at 4°C for 48 hours. The resulting dialysate was subjected to anion-exchange chromatography using a Toso's DEAE-TOYOPEARL 650 M column (column size: 5 x 6 ocm), from which was obtained a fraction having a nitrile hydratase activity through gradient elution with from 0 M to 0.5 M potassium chloride, using KPB as the developer. Next, the resulting active fraction was subjected to gel filtration chromatography, using Pharmacia's SUPERDEX200-26/60 as the carrier and using KPB containing 0.2 M potassium chloride as the developer, from which was recovered only the fraction having a nitrile hydratase activity. Then, the active fraction was subjected to hydrophobic chromatography using a Toso's TSK Gel Phenyl-5PW column (for HPLC). In this, the active ingredient was adsorbed to the carrier in the column that had been equilibrated with KPB containing ammonium sulfate at a concentration of 20 % saturation, and was then eluted through gradient elution with an eluent comprising ammonium sulfate, of which the concentration was decreased from 20 % saturation to 0 % saturation at a linear gradient, to obtain an active fraction.

In these chromatographic treatments, the nitrile hydratase activity in each fraction was determined in the manner

as mentioned below. Each fraction was suitably diluted with KPB, to which was added 1 % by weight of acrylonitrile and reacted at 10°C for 10 minutes. To the reaction mixture, added was an aqueous solution of 1 M phosphoric acid, which was the same amount as that of the reaction mixture, to thereby terminate the reaction. Then, the concentration of the thus-formed acrylamide was measured through HPLC analysis. In this, used was ULTRON 80HG (50 x 8 mm) as the HPLC column to which was applied a developer of an aqueous solution of 10 mM phosphoric acid. The amount of acrylamide formed was determined through the measurement of the absorbance at 220 nm.

The active fraction as obtained through the hydrophobic chromatography was subjected to SDS-PAGE under a reducing condition, which revealed the presence of two main polypeptide chains of 29 K daltons and 32 K daltons and three minor polypeptide chains of 45 K daltons or higher. SDS-PAGE of ordinary nitrile hydratases under a reducing condition generally gives two polypeptide chains of 30 +/- 3 K daltons. Each of the two main polypeptide chains of 29 K daltons and 32 K daltons existing in the SDS-PAGE gel was adsorbed onto a Bio Rad's PVDE membrane, using a Sartorius' semi-dry Electroblotter. From the PVDE membrane, only the part onto which the intended polypeptide chain had been adsorbed was cut out, and the N-terminal amino acid sequence of the polypeptide chain was sequenced using a Shimadzu's peptide sequencer, PSQ-I. As a result, it was found that the N-terminal amino acid sequence of the polypeptide chain of 29 K daltons is Thr-Glu-Asn-Ile-Leu-Arg-Lys that corresponds to from the 2nd to the 8th amino acid residues of the amino acid sequence of Sequence Number 1 in Sequence Listing, and that the N-terminal amino acid sequence of the polypeptide chain of 32 K daltons is Met-Asn-Gly-Val-Tyr-Asp-Val that corresponds to from 1st to 7th amino acid residues of the amino acid sequence of Sequence Number 2 in Sequence Listing.

Example 2:

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Isolation of Nitrile Hydratase Gene from Pseudonocardia thermophila JCM3095:

Cells of Pseudonocardia thermophila JCM3095 were incubated in the same manner as in Example 1. The culture was centrifuged (8000 x G, 30 minutes) to collect 2 g of wet cells therefrom. To these was added 40 ml of an aqueous solution of 50 mM EDTA-2Na (pH 8.0) containing 0.15 M NaCl to prepare a cell suspension, which was then boiled at 90°C for 10 minutes. The resulting suspension was cooled to 37°C, to which was added 100 mg of egg white lysozyme and kept at 37°C for 1 hour. Next, 30 mg of zymolylase of 20,000 U/mg was added to this, and kept at 37°C for 1 hour. Subsequently, 5 mg of proteinase K of 20 U/mg was added to this, and kept at 37°C for 1 hour. Further, 2 ml of 10 % SDS solution was added to this and kept at 650C for 1 hour, which was then immediately subjected to phenol/chloroform extraction. Precisely, 42 ml of phenol as saturated with TE (10 mM Tris-HCl buffer containing 1 mM EDTA-2Na; pH 8.0) was added to the reaction mixture and then gently stirred. This was centrifuged (3000 rpm, 10 minutes) to separate it into an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, added were 21 ml of the above-mentioned TE-saturated phenol and 21 ml of chloroform, and gently stirred. Then, this was again centrifuged (3000 mm, 10 minutes) to separate it into an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, added was 42 ml of chloroform, and gently stirred. Then, this was still again centrifuged (3000 rpm, 10 minutes) to separate it into an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, added were 4 ml of TE containing 1.1 M NaCl and 92 ml of ethanol, and then left as at room temperature for a while. Then, the yarnlike DNA thus precipitated was collected by winding it around a glass rod. This was dewatered through treatment with aqueous solutions of 70 %, 80 % and 90 % ethanol in that order, and then dried in air. Next, the thus-collected DNA was again dissolved in 40 ml of TE. To this was added 30 µg of RNase A, and kept at 37°C for 1 hour. Next, this was partially cleaved with a restriction endonuclease BamHI. The DNA thus partially cleaved was again purified through phenol/chloroform extraction followed by ethanol precipitation, and this was dissolved in TE to have a final concentration of 1.0 μg/μl.

On the basis of the N-terminal amino acid sequences of the polypeptide chains of 29 K daltons and 32 K daltons, that had been sequenced in Example 1, the following four PCR primers were prepared.

Primer 1: 5'-ACNGARAAYATNYTNMGNAA-3'

Primer 2: 5'-TTNCKNARNATRTTYTCNGT-3'

Primer 3: 5'-ATGAAYGGNGTNTAYGANGT-3'

Primer 4: 5'-ACNTCRTANACNCCRTTCAT-3'

The primer 1 of Sequence Number 5 in Sequence Listing, and the primer 2 of Sequence Number 6 in the same correspond to the respective complementary chains of the DNA chain that reversely corresponds to the N-terminal amino acid sequence of the polypeptide chain of 29 K daltons. The primer 3 of Sequence Number 7 in Sequence Listing, and the primer 4 of Sequence Number 8 in the same correspond to the respective complementary chains of the DNA chain that reversely corresponds to the N-terminal amino acid sequence of the polypeptide chain of 32 K daltons. In these, N indicates A, C, G or T.

3 μg of the chromosome DNA that had been partially cleaved hereinabove was subjected to PCR, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 100 μl in total, comprising 3 μg of the DNA, 200 ng of the primer 1, 200 ng of the primer 4 and 5 U of Taq DNA polymerase. The reaction No. 1 was comprised of 40 PCR cycles, in which one PCR cycle comprised thermal denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute and chain extension at 72°C for one minute. For PCR reaction No. 2, used was a reaction system of 100 μl in total, comprising 3 μg of the DNA, 200 ng of the primer 2, 200 ng of the primer 3 and 5 U of Taq DNA polymerase. The reaction No. 2 was comprised of 40 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 μl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the presence of the amplified DNA product of about 700 bp only in the reaction mixture obtained in the PCR reaction No. 2. This verified that, in Pseudonocardia thermophila, a gene of 32 K daltons and a gene of 29 K daltons exist while being adjacent to each other in that order from the upstream region of the 5'-terminal side of the combination of these genes.

A solution of the chromosome DNA that had been partially cleaved with BamHI hereinabove was subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 0.6 % by weight), and an agarose fragment comprising a DNA fragment of from about 1500 bp to about 4000 bp was cut out of the agarose gel. This agarose fragment (about 0.5 g) was finely pulverized, suspended in 5 ml of TE, and heated at 55°C for 1 hour, whereby the fragment was completely melted in TE. The resulting agarose melt was subjected to the same phenol/chloroform extraction and ethanol precipitation as in the above, thereby purifying the DNA fragment. The thus-purified DNA fragment was prepared in that manner in an amount of at lest 10 pmol, and inserted into the BamHI site existing in the multi-cloning site of a plasmid vector pUC18 (produced by Takara Shuzo), using a Takara Shuzo's DNA ligation kit. Prior to being used in this ligation, the pUC18 plasmid vector DNA was cleaved with a restriction endonuclease BamHI, then purified through phenol/chloroform extraction and ethanol precipitation, subjected to dephosphorylation of the 5'-terminal thereof with an alkali phosphatase (produced by Takara Shuzo), and again purified through phenol/chloroform extraction and ethanol precipitation. 20 ng of the pUC plasmid vector DNA that had been processed and purified in that manner was used in the ligation.

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With the Ihus-ligated DNA, competent cells of <u>Escherichia coli</u> HB101 (COMPETENT HIGH, produced by Toyobo Co., Ltd.) were transformed. About 10 µl of the liquid comprising the thus-transformed cells was applied onto an LB agar medium dishes (0.5 wt.% Bacto yeast-extract, 1 wt.% Bacto trypton, 0.5 wt.% sodium chloride, 1.5 wt.% agar; pH 7.5) containing 50 µg/ml of ampicillin and they were incubated at 37°C overnight. Thus were obtained a number of laboratory dishes each containing the medium that had from 100 to 1000 colonies/dish appeared thereon.

The plasmid library comprising the chromosome DNA was subjected to colony hybridization using, as the probe, the PCR-amplified DNA product that had been prepared hereinabove, and thus screened to select the clones comprising the intended nitrile hydratase gene.

Precisely, a nylon membrane, Amersham's Hybond-N was gently put onto each dish of the plasmid library, and after about one minute, this was gently peeled off. The thus-peeled membrane was then dipped in a denaturation liquid (aqueous solution of 0.5 M NaOH containing 1.5 M NaCI) for 7 minutes, and then processed with a neutralization liquid (an aqueous solution of 0.5 M Tris-HCl containing 1.5 M NaCl and 1 mM EDTA-2Na; pH 7.2) for 3 minutes. This neutralization was repeated twice. Next, this was washed once with 2 x SSC (1 x SSC is an aqueous solution of one liter comprising 8.76 g of sodium chloride and 4.41 g of sodium citrate), and then put on dry fitter paper, on which the membrane was dried in air. The membrane was then exposed to UV at 120 mJ/cm², by which the DNA was fixed onto the membrane. The thus-processed membranes were subjected to prehybridization, by dipping them in 30 ml/membrane of a hybridization buffer (5 x SSC further containing 1 wt.% skim milk, 0.1 wt.% N-lauroylsarcosine, 0.02 wt.% SDS, and 50 wt.% formamide) for 2 hours at 42°C. On the other hand, using the PCR-amplified DNA product that had been prepared hereinabove, as the template, and using a Boehringer Mannheim's DIG-DNA labeling kit, a fluorescence-labeled probe was prepared. 100 ng of the fluorescence-labeled probe and 300 ng of the pUC18 plasmid DNA were, after having been thermally denatured through boiling at 95°C for 10 minutes, transferred into 10 ml of the hybridization buffer along with the prehybridized membrane. After these were hybridized at 42°C for 24 hours therein, the membrane was washed twice with 150 ml of 2 x SSC containing 0.1 wt.% SDS, at room temperature. Next, this was again washed twice with 150 ml of 1 x SSC containing 0.1 wt.% SDS, at 68°C for 5 minutes. This was further washed with 100 ml of a buffer A (0.1 M maleic acid buffer containing 0.3 wt.% Tween 20 and 0.15 M NaCl; pH 7.5) for 5 minutes, and then blocked in a buffer B (0.1 M maleic acid buffer containing 0.3 wt.% Tween 20, 0.15 M NaCl and 1 wt.% skim milk; pH 7.5) at room temperature for 30 minutes. Next, this was washed twice with 300 ml of the buffer A at room temperature for 15 minutes, and then equilibrated in 60 ml of a buffer C (aqueous solution of 0.1 M Tris-HCl containing 0.1 M NaCl and 50 mM magnesium chloride; pH 9.5) for 5 minutes. This membrane was then dipped in 30 ml of a solution that had been prepared by diluting a luminescent substrate, Boehringer Mannheim's AMPPD 100-fold with the buffer C, at room temperature for 10 minutes, and transferred onto dry filter paper, which absorbed the excess AMPPD. The membrane thus processed according to the process mentioned hereinabove was

wrapped with a polyethylene film, which was then subjected to X-ray photography. On the resulting X-ray film, the position of the fluorescent signal appeared was confirmed. This verified the presence of one positive signal on the membrane, and the positive colony from which the membrane had been prepared was confirmed on the original laboratory dish.

The thus-confirmed positive colony was transplanted from the dish onto 10 ml of a liquid LB medium contained ampicillin, and incubated therein overnight at 37°C with stirring at 250 rpm. The plasmid DNA was extracted from the thus-incubated cells in an ordinary manner, then cleaved with a restriction endonuclease BamHI, and thereafter subjected to agarose gel electrophoresis (where the agarose concentration was 0.7 % by weight) to thereby determine the size of the insert fragment, resulting in that the size thereof was about 3.1 Kbp. This plasmid is referred to as pPT-BI (see Fig. 1). The full-length base sequence of the insert fragment was sequenced according to the primer extension method using ABI's sequencing kit and autosequencer 373A. As a result, it was verified that the insert fragment comprised open reading frames, one having a base sequence of 702 bp and the other having a base sequence of 618 bp, as bonded to each other in that order from the 5'-terminal side thereof. These open reading frames are referred to as ORF1 and ORF2. The four bases constituting the most 3'-terminal side of the ORF1 including the translation terminating codon, were the same as the four bases constituting the most 5'-terminal side of ORF2, which thus well corresponded to the result obtained through PCR. In addition, the amino acid sequence of the N-terminal side composed of seven amino acid residues, which had been presumed from the base sequence of ORF1, was completely the same as the amino acid sequence of the N-terminal side of the polypeptide chain of 32 K daltons that had been obtained in Example 1. This sequence region corresponds to the sequence of from the 1st to the 7th amino acid residues of the amino acid sequence of Sequence Number 2 in Sequence Listing. On the other hand, the amino acid sequence of from the 2nd to the 8th N-terminal side composed of seven amino acid residues, which had been presumed from the base sequence of ORF2, was completely the same as the amino acid sequence of the N-terminal side of 7 amino acid residues of the polypeptide chain of 29 K daltons that had been obtained in Example 1. This sequence region corresponds to the sequence of from the 2nd to the 8th amino acid residues of the amino acid sequence of Sequence Number 1 in Sequence Listing. In addition, high homology was admitted between the amino acid sequences of ORF1 and ORF2, and those of the β -subunit and α -subunit, respectively, of a different nitrile hydratase. From these, it was confirmed that the ORF1 is the β-subunit gene of the Pseudonocardia thermophila-derived nitrile hydratase and that the ORF2 is the α-subunit gene of the same.

30 Example 3:

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Construction of Transformant:

The transcription direction of the lac promoter on the pPT-B1 was completely the same as that of the ORF1 and that of the ORF2. The pPT-BI was cleaved with restriction endonucleases Xbal and NspV at its sites capable of being cleaved with these restriction endonucleases, these sites existing in the upstream region of the 5'-terminal side of the nitrile hydratase gene of the pPT-B1 and the pPT-B1 being able to be cleaved only at these sites, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 0.7 %), through which only the DNA fragment of about 4.6 Kbp was cut out of the plasmid. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of TE, and then heated at 55°C for 1 hour to thereby completely melt the agarose fragment. The resulting melt was subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the DNA fragment was purified. This was blunted, using a Takara Shuzo's DNA blunting kit, and then self-ligated using a Takara Shuzo's DNA ligation kit to thereby construct a plasmid pPT-DB1 (Fig. 2). This pPT-DB1 was introduced into competent cells of Escherichia coli HB101 (produced by Toyobo Co., Ltd.) whereby HB101 was transformed into a transformant MT-10822. In the transformant MT-10822, the nitrile hydratase gene was transcribed and translated via the lac promoter on the pUC18 existing therein. The transformant MT-10822 was deposited on February 7, 1996 and assigned deposit No. FERM P-15426 in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry of 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305, Japan, and transferred to deposit under the Budapest Treaty on January 10, 1997 and assigned deposit No. FERM BP-5785 in said depository.

Example 4:

Conversion (1) of Nitrile Compound into Amide Compound with Transformant:

100 ml of a liquid LB medium comprising 40 µg/ml of ferric sulfate 7-hydrate and 10 µg/ml of cobalt chloride dihydrate was put into a 500-ml Erlenmeyer flask equipped with baffles, and sterilized by autoclaving it at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. Then, one platinum loop

of the cells of MT-10822 that had been prepared in Example 3 were inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 130 rpm. The resulting culture was centrifuged (5000 G x 15 minutes) to separate only the cells therefrom, and the cells were suspended in 50 ml of a physiological saline solution. The resulting suspension was again centrifuged to separate the wet cells therefrom. 100 mg of the wet cells were suspended in 200 ml of an aqueous solution of 50 mM potassium phosphate (pH 7.0), to which was added 10 ml of acrylonitrile and reacted for 1 hour while gently stirring at 10°C. After the reaction, the reaction mixture was subjected to the same HPLC analysis as in Example 1, which verified the presence of only acrylamide in the reaction mixture and the absence of acrylonitrile and acrylic acid therein. Thus, the degree of conversion and the degree of selectivity in this reaction were 100 %.

Example 5:

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Conversion (2) of Nitrile Compound into Amide Compound with Transformant:

100 ml of a liquid LB medium comprising 40 μg/ml of ferric sulfate 7-hydrate and 10 μg/ml of cobalt chloride dihydrate was put into a 500-ml Erlenmeyer flask equipped with baffles, and sterilized by autoclaving it at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 μg/ml. Then, one platinum loop of the cells of MT-10822 that had been prepared in Example 3 were inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 130 rpm. The resulting culture was centrifuged (5000 G x 15 minutes) to separate only the cells therefrom. The cells were suspended in 50 ml of a physiological saline solution. The resulting suspension was again centrifuged to separate the wet cells therefrom. 100 mg of the wet cells were suspended in 200 ml of an aqueous solution of 50 mM potassium phosphate (pH 7.0), to which was added 50 ml of methacrylonitrile and reacted for 2 hours with gently stirring at 10°C. After the reaction, the reaction mixture was subjected to the same HPLC analysis as in Example 1, which verified the presence of only methacrylamide in the reaction mixture and the absence of methacrylonitrile and methacrylic acid therein. Thus, the degree of conversion and the degree of selectivity in this reaction were 100 %.

Example 6:

The Mutant (1) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Met for the 6th Leu in the α-subunit region in the plasmid DNA pPT-DB1 that had been prepared in Example 3. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation using a Takara Shuzo's "LA PCR in vitro mutagenesis Kit", in the manner mentioned below. The "LA PCR in vitro mutagenesis Kit" is hereinunder referred to as the kit. In the process for the mutation mentioned below, the kit was handled on the basis of the principle thereof and in accordance with the manufacturer's instructions for the kit.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 µg/ml. One platinum loop of the cells of MT-10822 that had been prepared in Example 3 were inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA pPT-DB1 through alkali SDS extraction.

One µg of the plasmid DNA pPT-DB1 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 μl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 9 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. From these PCR reaction mixtures, removed were the excess primers and dNTP, using Takara Shuzo's Microcon 100. To each was added TE to prepare TE solutions of 50 μ l each. Annealing solution of 47.5 μ l in total was prepared, containing 0.5 μ of both of the above TE solutions.

For the basic composition of the annealing solution, referred to were the manufacturer's instructions for the kit. This solution was then thermally denatured at 98°C for 10 minutes, then cooled to 37°C at a constant cooling rate over a period of 60 minutes, and thereafter kept at 37°C for 15 minutes. Thus, the intended annealing was finished. 0.5 µl of TAKARA LA Taq was added to the thus-annealed solution, and then heated at 72°C for 3 minutes. Thus, the formation of heterologous double-stranded DNA was completed, which was then subjected to PCR reaction No. 3. Precisely, for the PCR reaction No. 3, used was a reaction system of 50 μl in total, comprising 48 μl of said heterologus doublestranded DNA solution,50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing). The PCR reaction No. 3 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. After PCR, 5 µl of the reaction mixture obtained in the PCR reaction No. 3 was subjected to agarose gel electrophoresis (using a low-meltingpoint agarose, Sigma's Type VII at an agarose concentration of 0.8 % by weight), thereby detecting the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA product of about 2.0 Kbp in the PCR reaction No. 3. From the PCR reaction mixture, taken out was only the DNA fragment of about 2.0 Kbp. This fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of TE, and heated at 55°C for 1 hour, whereby the fragment was completely melted. The resulting melt was then subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the DNA fragment was purified. Finally, this was dissolved in 10 µl of TE. The amplified DNA fragment of about 20 Kbp thus purified was cleaved with restriction endonucleases EcoRI and HindIII, and then subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the DNA fragment was purified. Finally, this was dissolved in 10 µl of TE. On the other hand, the plasmid pPT-DB1 was cleaved with restriction endonucleases EcoRI and HindIII at their cleaving sites, then subjected to agarose get electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 0.7 %), through which only the DNA fragment of about 2.7 Kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of TE, and heated at 55°C for 1 hour, whereby the fragment was completely melted in TE. The resulting melt was subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the DNA fragment was purified. Finally, this was dissolved in 10 µl of TE. The amplified DNA product and the pPT-DB1 fragment thus prepared in the above were ligated together, using a Takara Shuzo's DNA ligation kit, with which competent cells of Escherichia coli HB101 (produced by Toyobo) was transformed. Thus was prepared an E. coli bank.

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10 ml of a liquid LB medium comprising 40 μ g/ml of ferric sulfate 7-hydrate and 10 μ g/ml of cobalt chloride dihydrate (this medium is hereinafter referred to as an activity expressionmedium) was put into a 30-ml test tube, and sterilized by autoclaving it at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 μ g/ml. Then, one platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube and centrifuged (at 15,000 rpm for 15 minutes) to thereby separate the cells from the culture. The cells were suspended in 200 μ l of a potassium phosphate buffer (pH 7.0), to which was added 1% by weight of acrylonitrile and reacted at 10°C for 2 minutes. To the reaction mixture, added was the same amount, as that of the reaction mixture, of an aqueous solution of 1 M phosphoric acid, with which the reaction was terminated. The concentration of the acrylamide thus produced in the reaction mixture was determined through the same HPLC analysis as in Example 1. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced through the same primer extension as in Example 2, using the same ABI's sequencing kit and auto-sequencer 373A. The results are shown in Table 1, in which it is known that the 6th Leu in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Met.

Table 1

Clone Number	Mutated Site (in α-subunit)	Change in Amin	o Acid Sequence	Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 1	6th position in α- subunit	Leu	Met	CTG	ATG

Example 7:

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The Mutant (2) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Thr for the 6th Leu in the α -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 13 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 2, in which it is known that the 6th Leu in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Thr.

Table 2

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
	·	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 2	6th position in α- subunit	Leu	Thr	CTG	ACG

Example 8:

The Mutant (3) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Ala for the 6th Leu in the α -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 14 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing)

and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 3, in which it is known that the 6th Leu in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

Table 3

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 3	6th position in α- subunit	Leu	Ala	CTG	GCG

Example 9:

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The Mutant (4) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Val for the 6th Leu in the α -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 15 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction

to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 4, in which it is known that the 6th Leu in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

Table 4

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
,		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 4	6th position in α- subunit	Leu	Val	CTG	GTG

Example 10:

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The Mutant (5) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Val for the 19th Ala in the α -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 16 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 5, in which it is known that the 19th Ala in the α-subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

Table 5

Clone Number	Mutated Site (in α-subunit)	Change in Amin	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution	
No. 5	19th position in α-subunit	Ala	Val	GCG	GTG	

Example 11:

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The Mutant (6) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Leu for the 38th Met in the α -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 17 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one mI of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 6, in which it is known that the 38th Met in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

Table 6

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 6	38th position in α-subunit	Met	Leu	ATG	TTG

Example 12:

The Mutant (7) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Ser for the 77th Thr in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 18 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing)

and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 μ l of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 7, in which it is known that the 77th Thr in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Ser.

Table 7

			(5,5 .		
Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 7	77th position in α-subunit	Thr	Ser	ACC	TCC

Example 13:

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The Mutant (8) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Ala for the 90th Gly in the α -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 19 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of

each clone was sequenced in the same manner as in Example 2. The results are shown in Table 8, in which it is known that the 90th Gly in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

Table 8

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 8	90th position in α-subunit	Gly	Ala	GGC	GCC

Example 14:

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The Mutant (9) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Ala for the 102nd Val in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 20 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 pm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 9, in which it is known that the 102nd Val in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

Table 9

Clone Number	Mutated Site (in α-subunit)	Change in Amin	o Acid Sequence	Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 9	102nd position in α-subunit	Val	Ala	GTC	GCC

Example 15:

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The Mutant (10) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with lie for the 106th Val in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 21 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 10, in which it is known that the 106th Val in the α-subunit of the nitrile hydratase from the clone shown therein was substituted with Ile.

Table 10

Clone Number	Clone Number Mutated Site (in α-subunit) Change in Amino Acid Sequence Change in Base Se		Change in Amino Acid Sequence		` ' '		Base Sequence
		Before Substitution	After Substitution	Before Substitution	After Substitution		
No. 10	106th position in α-subunit	Val	lle	GTC	ATC		

Example 16:

The Mutant (11) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Tyr for the 126th Phe in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 22 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing)

and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 11, in which it is known that the 126th Phe in the α-subunit of the nitrile hydratase from the clone shown therein was substituted with Tyr.

Table 11

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in E	Base Sequence
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 11	126th position in α-subunit	Phe	Tyr	TTC	TAC

Example 17:

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The Mutant (12) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Glu for the 130th Gln in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 23 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of

each clone was sequenced in the same manner as in Example 2. The results are shown in Table 12, in which it is known that the 130th Gln in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Glu.

Table 12

Clone Number	Mutated Site (in α-subunit)	Change in Amin	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution	
No. 12	130th position in α-subunit	Gln	Glu	CAG	GAG	

Example 18:

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The Mutant (13) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Val for the 142nd Leu in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 24 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 13, in which it is known that the 142nd Leu in the α-subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

Table 13

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 13	142nd position in α-subunit	Leu	Val	CTG	GTG

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Example 19:

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The Mutant (14) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Asp for the 146th Glu in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 25 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one mI of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 14, in which it is known that the 146th Glu in the α-subunit of the nitrile hydratase from the clone shown therein was substituted with Asp.

Table 14

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in E	Base Sequence
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 14	146th position in α-subunit	Glu	Asp	GAG	GAC

Example 20:

The Mutant (15) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Thr for the 187th Ala in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 26 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing)

and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, $5\,\mu$ I of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an \underline{E} , \underline{coli} bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 15, in which it is known that the 187th Ala in the α-subunit of the nitrile hydratase from the clone shown therein was substituted with Thr.

Table 15

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Clone Number	Mutated Site (in α-subunit)	` •		Change in Base Sequence	
·		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 15	187th position in α-subunit	Ala	Thr	GCC	ACC

Example 21:

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The Mutant (16) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Leu for the 194th Ser in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 27 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the piasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of

each clone was sequenced in the same manner as in Example 2. The results are shown in Table 16, in which it is known that the 194th Ser in the α -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

Table 16

Clone Number	Mutated Site (in α-subunit)	Change in Amin	o Acid Sequence	Change in E	Base Sequence
•		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 16	194th position in α-subunit	Ser	Leu	TCG	TTG

Example 22:

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The Mutant (17) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Glu for the 203rd Ala in the α-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 28 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 17, in which it is known that the 203rd Ala in the α-subunit of the nitrile hydratase from the clone shown therein was substituted with Glu.

Table 17

Clone Number	Mutated Site (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 17	203rd position in α-subunit	Ala	Glu	GCG	GAG

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Example 23:

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The Mutant (18) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Val for the 20th Ala in the β -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 29 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 18, in which it is known that the 20th Ala in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

Table 18

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Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in E	Base Sequence
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 18	20th position in β-subunit	Ala	Val	GCG	GTG

Example 24:

The Mutant (19) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Asn for the 21st Asp in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 30 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing)

and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, $5\,\mu$ I of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an \underline{E} , \underline{coli} bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 19, in which it is known that the 21st Asp in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Asn.

Table 19

Clone Number	Mutated Site (in β-subunit)	Change in Amin	o Acid Sequence	Change in E	Base Sequence
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 19	21st position in β-subunit	Asp	Asn	GAC	AAC

Example 25:

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The Mutant (20) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Asp for the 108th Glu in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 31 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 pm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of

each clone was sequenced in the same manner as in Example 2. The results are shown in Table 20, in which it is known that the 108th Glu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Asp.

Table 20

Clone Number	Mutated Site (in	Change in Amin	o Acid Sequence	Change in E	Base Sequence
	β-subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 20	108th position in β-subunit	Glu	Asp	GAG	GAT

Example 26:

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The Mutant (21) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Pro for the 108th Glu in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 32 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 21, in which it is known that the 108th Glu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Pro.

Table 21

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 21	108th position in β-subunit	Glu	Pro	GAG	CCG

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Example 27:

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The Mutant (22) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Ser for the 108th Glu in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 33 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 pm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 22, in which it is known that the 108th Glu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Ser.

Table 22

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in E	ase Sequence
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 22	108th position in β-subunit	Glu	Ser	GAG	TCG

Example 28:

The Mutant (23) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Arg for the 108th Glu in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 34 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the com-

position of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, $5\,\mu$ I of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 23, in which it is known that the 108th Glu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Arg.

Table 23

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
·		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 23	108th position in β-subunit	Glu	Arg	GAG	CGG

Example 29:

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The Mutant (24) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Cys for the 108th Glu in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 35 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 pm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 24, in which it is known that the 108th Glu in the β -subunit of the nitrile hydratase from the clone shown therein was substituted with Cys.

Table 24

Clone Number	Mutated Site (in β-subunit) . Change in Amir		Change in Amino Acid Sequence		Base Sequence
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 24	108th position in β-subunit	Glu	Cys	GAG	TGC

Example 30:

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The Mutant (25) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Leu for the 108th Glu in the β -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 μ g of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 μ l in total, comprising 50 pmols of the primer having the sequence of Sequence Number 36 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 μ l in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 μ l of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by welght), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E, coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 25, in which it is known that the 108th Glu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

Table 25

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Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		BeforeSubstitution	After Substitution	Before Substitution	After Substitution
No. 25	108th position in β-subunit	Glu	Leu	GAG	CTG

Example 31:

The Mutant (26) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Thr for the 108th Glu in the β-subunit region in the plasmid DNA pPT-

DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 37 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 26, in which it is known that the 108th Glu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Thr.

Table 26

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 26	108th position in β-subunit	Glu	Thr	GAG	ACG

Example 32:

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The Mutant (27) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Asp for the 200th Ala in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 38 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA

in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 27, in which it is known that the 200th Ala in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Asp.

Table 27

15	Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence		
			Before Substitution	After Substitution	Before Substitution	After Substitution	
20	No. 27	200th position in β-subunit	Ala	Asp	GCC	GAC	

Example 33:

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The Mutant (28) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with the for the 200th Ala in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 39 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 28, in which it is known that the 200th Ala in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with lie.

Table 28

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence		
		Before Substitution	After Substitution	Before Substitution	After Substitution	
No. 28	200th position in β-subunit	Ala	lle	GCC	ATC	

Example 34:

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The Mutant (29) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Val for the 200th Ala in the β -subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 40 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 29, in which it is known that the 200th Ala in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

Table 29

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Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence		
	,	Before Substitution	After Substitution	Before Substitution	After Substitution	
No. 29	200th position in β-subunit	Ala	Val	GCC	GTC	

Example 35:

The Mutant (30) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Glu for the 200th Ala in the β-subunit region in the plasmid DNA pPT-

DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 41 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 30, in which it is known that the 200th Ala in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Glu.

Table 30

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 30	200th position in β-subunit	Ala	Glu	GCC	GAG

Example 36:

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The Mutant (31) with the Partial Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate the substitution with Tyr for the 212nd Ser in the β-subunit region in the plasmid DNA pPT-DB1. Using it as the template, the plasmid DNA pPT-DB1 was subjected to site-specific mutation in the same manner as in Example 6.

In this, 1 µg of the plasmid DNA pPT-DB1 that had been prepared in Example 6 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 42 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA

in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 31, in which it is known that the 212nd Ser in the β -subunit of the nitrile hydratase from the clone shown therein was substituted with Tyr.

Table 31

Clone Number Mutated Site (in		Change in Amino Acid Sequence		Change in Base Sequence		
	β-subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution	
No. 31	212nd position in β-subunit	Ser	Tyr	TCC	TAC	

Exmple 37:

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The Mutant (32) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 5 (where the 19thAla in the α -subunit was substituted with Val) and that from the clone No. 11 (where the 126th Phe in the α -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 µg/ml. One platinum loop of the clone No. 11 that had been prepared in Example 16 was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA of the clone No. 11 through alkali SDS extraction.

One µg of the plasmid DNA of the clone No. 11 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 16 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction

to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 32, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 19th Ala in the α -subunit in the wild nitrile hydratase was substituted with Val and the 126th Phe in the same was substituted with Tyr.

Table 32

Clone Number	Mutated Sites (in α-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 32	19th position in α-subunit	Ala	Val	GCG	GTG
	126th position in α-subunit	Phe	Tyr	TTC	TAC

Exmple 38:

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The Mutant (33) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 1 (where the 6th Leu in the α -subunit was substituted with Met) and that from the clone No. 32 (where the 19th Ala in the α -subunit was substituted with Val and the 126th Phe in the α -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 µg/ml. One platinum loop of the clone No. 32 that had been prepared in Example 37 was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA of the clone No. 32 through alkali SDS extraction.

One μg of the plasmid DNA of the clone No. 32 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 μ l in total, comprising 50 pmols of the primer having the sequence of Sequence Number 9 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 μ l in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 μ l of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E, coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 33, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 6th Leu in the α -subunit in the wild nitrile hydratase was substituted with Met , the 19th Ala in the same was substituted with Val, and the 126th Phe in the same was substituted with Tyr.

Table 33

Clone Number	Mutated Sites	Change in Amino Acid Sequence		Change in Base Sequence		
		Wild Type	Mutant	Wild Type	Mutant	
No. 33	6th position in α-subunit	Leu	Met	ста	ATG	
	19th position in α-subunit	Ala	Val	GCG	GTG	
	126th position in α-subunit	Phe	Tyr	ттс	TAC	

Exmple 39:

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The Mutant (34) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 2 (where the 6th Leu in the α -subunit was substituted with Thr) and that from the clone No. 32 (where the 19th Ala in the α -subunit was substituted with Val and the 126th Phe in the α -subunit was substituted with Tyr) still had the nitrile hydratase activity.

One µg of the plasmid DNA of the clone No. 32 that had been prepared in Example 38 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 13 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 34, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 6th Leu in the α -subunit in the wild nitrile hydratase was substituted with Thr , the 19th Ala in the same was substituted with Val, and the 126th Phe in the same was substituted with Tyr.

Table 34

Clone Number	Mutated Sites	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 34	6th position in α-subunit	Leu	Thr	CTG	ATG
	19th position in α-subunit	Ala	Val	GCG	GTG
	126th position in α-subunit	Phe	Tyr	ттс	TAC

Exmple 40:

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The Mutant (35) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 3 (where the 6th Leu in the α -subunit was substituted with Ala) and that from the clone No. 32 (where the 19th Ala in the α -subunit was substituted with Val and the 126th Phe in the α -subunit was substituted with Tyr) still had the nitrile hydratase activity.

One µg of the plasmid DNA of the clone No. 32 that had been prepared in Example 38 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 14 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E. coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 35, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 6th Leu in the α -subunit in the wild nitrile hydratase was substituted with Ala , the 19th Ala in the same was substituted with Val, and the 126th Phe in the same was substituted with Tyr.

Table 35

Clone Number	Mutated Sites	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 35	6th position in α-subunit	Leu	Ala	ста	GCG
	19th position in α -subunit	Ala	Val	GCG	GTG
	126th position in α-subunit	Phe	, Tyr	ттс	TAC

Exmple 41:

The Mutant (36) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 20(where the 108th Glu in the β -subunit was substituted with Asp) and that from the clone No. 31 (where the 212nd Ser in the β -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 µg/ml. One platinum loop of the clone No. 31 that had been prepared in Example 36 was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifugal at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA of

the clone No. 31 through alkali SDS extraction.

One μg of the plasmid DNA of the clone No. 31 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 μ l in total, comprising 50 pmols of the primer having the sequence of Sequence Number 31 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 μ l in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 μ l of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E, E coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 36, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 108th Glu in the β -subunit in the wild nitrile hydratase was substituted with Asp and the 212nd Ser in the same was substituted with Tyr.

Table 36

Clone Number	Mutated Sites	Change in Amino A	Acid Sequence	Change in Base	e Sequence
		Wild Type	Mutant	Wild Type	Mutant
No. 36	108th position in β-subunit	Glu	Asp	GAG	GAT
	212nd position in β-subunit	Ser	Tyr	TCC	TAC

Exmple 42:

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The Mutant (37) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 23(where the 108th Glu in the β -subunit was substituted with Arg) and that from the clone No. 31 (where the 212nd Ser in the β -subunit was substituted with Tyr) still had the nitrile hydratase activity.

One µg of the plasmid DNA of the clone No. 31 that had benn prepared in the Example 41 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 34 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one mI of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 37, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 108th Glu in the β -subunit in the wild nitrile hydratase was substituted with Arg and the 212nd Ser in the same was substituted with Tyr.

Table 37

Clone Number	Mutated Sites	Change in Amino A	Acid Sequence	Change in Base	e Sequence
	•	Wild Type	Mutant	Wild Type	Mutant
No. 37	108th position in β-subunit	Glu	Arg	GAG	CGG
	212nd position in β-subunit	Ser	Tyr	TCC	TAC

Exmple 43:

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The Mutant (38) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 27(where the 200th Ala in the β-subunit was substituted with Asp) and that from the clone No. 31 (where the 212nd Ser in the β-subunit was substituted with Tyr) still had the nitrile hydratase activity.

One μg of the plasmid DNA of the clone No. 31 that had benn prepared in the Example 41 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 μl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 38 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 μl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 μl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 38, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 200th Ala in the β -subunit in the wild nitrile hydratase was substituted with Asp and the 212nd Ser in the same was substituted with Tyr.

Table 38

Clone Number	Mutated Sites	Change in Amino A	Acid Sequence	Change in Base Sequence		
		Wild Type	Mutant	Wild Type	Mutant	
No. 38	200th position in β-subunit	Ala	Asp	GCC	GAC	
	212nd position in β-subunit	Ser	Tyr	TCC	TAC	

Exmple 44:

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The Mutant (39) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 30(where the 200th Ala in the β -subunit was substituted with Glu) and that from the clone No. 31 (where the 212nd Ser in the β -subunit was substituted with Tyr) still had the nitrile hydratase activity.

One µg of the plasmid DNA of the clone No. 31 that had benn prepared in the Example 41 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 µl in total, comprising 50 pmols of the primer having the sequence of Sequence Number 41 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 µl in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 µl of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an <u>E. coli</u> bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 39, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 200th Ala in the β -subunit in the wild nitrile hydratase was substituted with Glu and the 212nd Ser in the same was substituted with Tyr.

Table 39

Clone Number	Mutated Sites	Change in Amino	Acid Sequence	Change in Bas	e Sequence
		Wild Type	Mutant	Wild Type	Mutant
No. 39	200th position in β-subunit	Ala	Glu	GCC	GAG
	212nd position in β-subunit	Ser	Tyr	тсс	TAC

Exmple 45:

The Mutant (40) with the Multi-Substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising both of the mutated position from the clone No. 23(where the 108th Glu in the β -subunit was substituted with Arg) and that from the clone No. 39 (where the 200nd Ala in the β -subunit was substituted with Glu and the 212nd Ser in the same was



substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 µg/ml. One platinum loop of the clone No. 39 that had been prepared in Example 44 was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA of the clone No. 39 through alkali SDS extraction.

One μg of the plasmid DNA of the clone No. 39 was subjected to PCR of two different types, using it as the template. Precisely, for PCR reaction No. 1, used was a reaction system of 50 μ l in total, comprising 50 pmols of the primer having the sequence of Sequence Number 34 in Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence of Sequence Number 10 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 1 was comprised of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation at 98°C for 15 seconds, annealing at 55°C for 30 seconds and chain extension at 72°C for 120 seconds. For PCR reaction No. 2, used was a reaction system of 50 μ l in total, comprising 50 pmols of an MUT 4 primer (having the sequence of Sequence Number 11 in Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence of Sequence Number 12 in Sequence Listing) (for the composition of this system, referred to were the manufacturer's instructions for the kit). The reaction No. 2 was comprised of 25 PCR cycles, in which one PCR cycle was the same as that in the reaction No. 1. After PCR, 5 μ l of the reaction mixture obtained in each of the reaction Nos. 1 and 2 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0 % by weight), thereby detecting, if any, the product resulting from the amplification of the DNA in the reaction mixture. This detection revealed the production of the amplified DNA products in the both PCR reactions. Using these products, an E, coli bank was prepared in the same manner as in Example 6.

One platinum loop of any one of five clones as unlimitedly selected from the <u>E. coli</u> bank was inoculated on 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 mm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was measured in the same manner as in Example 6. As a result, the formation of acrylamide was detected in four of the five clones, which verifies that the four clones had a nitrile hydratase activity.

From one ml of each of the cultures of the active four clones, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of each clone. Next, the base sequence of the nitrile hydratase structural gene of each clone was sequenced in the same manner as in Example 2. The results are shown in Table 40, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 108th Glu in the β -subunit in the wild nitrile hydratase was substituted with Arg ,the 200th Ala in the same was substituted with Glu, and the 212nd Ser in the same was substituted with Tyr.

Table 40

Clone Number	Mutated Sites	Change in Amino	Acid Sequence	Change in Base	e Sequence
!		` Wild Type	Mutant	Wild Type	Mutant
No. 40	108th position in β-subunit	Glu	Arg	GAG	CGG
	200th position in β-subunit	. Ala	Glu	GCC	GAG
	212nd position in β-subunit	Ser	Туг	TCC	TAC

Example 46:

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The Mutant (41) with the multi-substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising bot of the mutated position from the clone No. 34 and that from the clone No. 36 still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 µg/ml. Two same media of that type were prepared herein. One platinum loop of the clone No. 34 that had been prepared in Example 39 and one platinum loop of the clone No. 36 that had been prepared in Example 41 were separately inoculated on these media, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. The resulting cultures of one ml each were separately put into suitable centrifugal tubes, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the cultures. From the respective cells were extracted the plasmid DNA of the clone No. 34 and the plasmid DNA of the clone No.

36 through alkali SDS extraction.

The plasmid DNA from the clone No. 36 was cleaved with restriction endonucleases EcoRI and Notl at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 1.0 %), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No.34 was cleaved with restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 Kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of TE, and heated at 55°C for 1 hour, whereby the fragments were completely melted. The resulting melts were separately subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the fragments were purified. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the clone No. 36 and the DNA fragment of about 3.8 Kbp obtained from the clone No. 34 were ligated together, using a Takara Shuzo's DNA ligation kit, to construct a plasmid pPT-DB41. This plasmid DNA pPT-DB41 was introduced into competent cells of Escherichia coli HB101 (produced by Toyobo). Thus was obtained an E. coli clone No. 41.

One platinum loop of the cells of the E. coli clone No. 41 were inoculated onto 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was determined in the same manner as in Example 6. As a result, the formation of acrylamide was detected, which verifies that the clone No. 41 still had the nitrile hydratase activity.

From one ml of the culture, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of the clone. Next, the base sequence of the nitrile hydratase structural gene of the clone No. 41 was sequenced in the same manner as in Example 2. The results are shown in Table 41, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 6th Leu in the a-subunit in the wild nitrile hydratase was substituted with Thr, the 19th Ala in the same was substituted with Val, the 126th Phe in the same was substituted with Tyr, while the 108th Glu in the β-subunit in the wild nitrile hydratase was substituted with Asp, and the 212nd Ser in the same was substituted with Tyr.

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Table 41

Clone Number	Mutated Sites	Change in Amino	Acid Sequence	Change in Base Sequence			
·		Wild Type	Mutant	Wild Type	Mutant		
No. 41	6th position in α-subunit	Leu	Thr	CTG	ACG		
	19th position in α -subunit	Ala	Val	GCG	GTG		
•	126th position in α-subunit	Phe	Tyr	TTC	TAC		
	108th position in β-subunit	Glu	Asp	GAG	GAT		
	212nd position in β-subunit	Ser	Tyr	тсс	TAC		

Example 47:

The Mutant (42) with the multi-substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising bot of the mutated position from the clone No. 34 and that from the clone No. 37 still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 μg/ml. One platinum loop of the clone No. 37 that had been prepared in Example 42 was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA of the clone No. 37 through alkali SDS extraction.

The plasmid DNA from the clone No. 37 was cleaved with restriction endonucleases EcoRl and Notl at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 1.0 %), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No.34 that had been prepared in the Example 46 was cleaved

with restriction endonucleases EcoRl and Notl at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 Kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of TE, and heated at 55°C for 1 hour, whereby the fragments were completely melted. The resulting melts were separately subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the fragments were purified. Finally, these were separately dissolved in 10 μ l of TE.

The DNA fragment of about 770 bp obtained from the clone No. 37 and the DNA fragment of about 3.8 Kbp obtained from the clone No. 34 were ligated together, using a Takara Shuzo's DNA ligation kit, to construct a plasmid pPT-DB42. This plasmid DNA pPT-DB42 was introduced into competent cells of <u>Escherichia coli</u> HB101 (produced by Toyobo). Thus was obtained an E. coli clone No. 42.

One platinum loop of the cells of the <u>E. coli</u> clone No. 42 were inoculated onto 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was determined in the same manner as in Example 6. As a result, the formation of acrylamide was detected, which verifies that the clone No. 42 still had the nitrile hydratase activity.

From one ml of the culture, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of the clone. Next, the base sequence of the nitrile hydratase structural gene of the clone No. 42 was sequenced in the same manner as in Example 2. The results are shown in Table 42, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 6th Leu in the α -subunit in the wild nitrile hydratase was substituted with Thr, the 19th Ala in the same was substituted with Val, the 126th Phe in the same was substituted with Tyr, while the 108th Glu in the β -subunit in the wild nitrile hydratase was substituted with Arg, and the 212nd Ser in the same was substituted with Tyr.

Table 42

Clone Number	Mutated Sites	Change in Amino	Acid Sequence	Change in Base Sequence			
		Wild Type	Mutant	Wild Type	Mutant		
No. 42	6th position in α-subunit	Leu	Thr	CTG	ACG		
	19th position in α-subunit	Ala	· Val	GCG	GTG		
	126th position in α-subunit	Phe	Tyr	ттс	TAC		
	108th position in β-subunit	Glu	Arg	GAG	CGG		
	212nd position in β-subunit	Ser	Tyr	тсс	TAC		

Example 48:

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The Mutant (43) with the multi-substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising bot of the mutated position from the clone No. 34 and that from the clone No. 39 still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 μ g/ml. One platinum loop of the clone No. 39 that had been prepared in Example 44 was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA of the clone No. 39 through alkali SDS extraction.

The plasmid DNA from the clone No. 39 was cleaved with restriction endonucleases EcoRl and Notl at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 1.0 %), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No.34 that had been prepared in the Example 46 was cleaved with restriction endonucleases EcoRl and Notl at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 Kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of TE, and heated at 55°C for 1 hour, whereby the

fragments were completely melted. The resulting melts were separately subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the fragments were purified. Finally, these were separately dissolved in 10 μ l of TE.

The DNA fragment of about 770 bp obtained from the clone No. 39 and the DNA fragment of about 3.8 Kbp obtained from the clone No. 34 were ligated together, using a Takara Shuzo's DNA ligation kit, to construct a plasmid pPT-DB43. This plasmid DNA pPT-DB43 was introduced into competent cells of <u>Escherichia coli</u> HB101 (produced by Toyobo). Thus was obtained an <u>E. coli</u> clone No. 43.

One platinum loop of the cells of the <u>E. coli</u> clone No. 43 were inoculated onto 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was determined in the same manner as in Example 6. As a result, the formation of acrylamide was detected, which verifies that the clone No. 43 still had the nitrile hydratase activity.

From one mI of the culture, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of the clone. Next, the base sequence of the nitrile hydratase structural gene of the clone No. 43 was sequenced in the same manner as in Example 2. The results are shown in Table 43, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 6th Leu in the α -subunit in the wild nitrile hydratase was substituted with Thr, the 19th Ala in the same was substituted with Val, the 126th Phe in the same was substituted with Tyr, while the 200th Ala in the β -subunit in the wild nitrile hydratase was substituted with Glu, and the 212nd Ser in the same was substituted with Tyr.

Table 43

Clone Number	Mutated Sites	Change in Amino A	Acid Sequence	Change in Base	e Sequence
		Wild Type	Mutant	Wild Type	Mutant
No. 43	6th position in α-subunit	Leu	Thr	CTG	ACG
	19th position in α-subunit	Ala	Val	GCG	GTG
	126th position in α-subunit	Phe	Tyr	TTC	TAC
	200th position in β-subunit	. Ala	Glu	GCC	GAG
	212nd position in β-subunit	Ser	Tyr	тсс	TAC

Example 49:

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The Mutant (44) with the multi-substituted Amino Acid Sequence Having Nitrile Hydratase Activity:

This is to demonstrate that the mutant with the multi-substituted amino acid sequence comprising bot of the mutated position from the clone No. 34 and that from the clone No. 40 still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30-ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. To this medium, added was ampicillin to have a final concentration of 100 µg/ml. One platinum loop of the clone No. 40 that had been prepared in Example 45 was inoculated on the medium, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and centrifuged at 15,000 rpm for 5 minutes to separate the cells from the culture. From the cells was extracted the plasmid DNA of the clone No. 40 through alkali SDS extraction.

The plasmid DNA from the clone No. 40 was cleaved with restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 1.0 %), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No.34 that had been prepared in the Example 46 was cleaved with restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using a low-melting-point agarose, Sigma's Type VII, at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 Kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of TE, and heated at 55°C for 1 hour, whereby the fragments were completely melted. The resulting melts were separately subjected to the same phenol/chloroform extraction and ethanol precipitation as in Example 2, through which the fragments were purified. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the clone No. 40 and the DNA fragment of about 3.8 Kbp obtained



from the clone No. 34 were ligated together, using a Takara Shuzo's DNA ligation kit, to construct a plasmid pPT-DB44. This plasmid DNA pPT-DB44 was introduced into competent cells of <u>Escherichia coli</u> HB101 (produced by Toyobo). Thus was obtained an <u>E. coli</u> clone No. 44.

One platinum loop of the cells of the <u>E. coli</u> clone No. 44 were inoculated onto 10 ml of the same activity expression medium as that used in Example 6, and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. One ml of the resulting culture was put into a suitable centrifugal tube, and its nitrile hydratase activity was determined in the same manner as in Example 6. As a result, the formation of acrylamide was detected, which verifies that the clone No. 44 still had the nitrile hydratase activity.

From one mI of the culture, which had remained herein without being used for the detection of the nitrile hydratase activity thereof, the cells were separated and subjected to alkali SDS extraction to thereby extract the plasmid DNA of the clone. Next, the base sequence of the nitrile hydratase structural gene of the clone No. 44 was sequenced in the same manner as in Example 2. The results are shown in Table 44, in which it is known that, in the amino acid sequence of the plasmid DNA as prepared herein, the 6th Leu in the α -subunit in the wild nitrile hydratase was substituted with Thr, the 19th Ala in the same was substituted with VaI, the 126th Phe in the same was substituted with Tyr, while the 108th Glu in the β -subunit in the wild nitrile hydratase was substituted with Arg, the 200th Ala in the same was substituted with Glu, and the 212nd Ser in the same was substituted with Tyr.

Table 44

Clone Number	Mutated Sites	Change in Amino	Acid Sequence	Change in Base Sequence			
		Wild Type	Mutant	Wild Type	Mutant		
No. 44	6th position in α-subunit	Leu	Thr	CTG	ACG		
	19th position in α-subunit	Ala	Val	GCG	GTG		
	126th position in α-subunit	Phe	Tyr	TTC	TAC		
	108th position in β-subunit	Glu	Arg	GAG	cgg		
	200th position in β-subunit	Ala	Glu	GCC	GAG		
	212nd position in β-subunit	Ser	Tyr	тсс	TAC		

According to the present invention, there is provided the amino acid sequence and base sequence of a <u>Pseudonocardia thermophila</u>-derived nitrile hydratase. Also provided are a method for changing its amino acid sequence and base sequence without substantially changing the functions of said nitrite hydratase, and nitrile hydratases having a base sequence and an amino acid sequence as changed on the basis of said method. Further provided are a recombinant plasmid having the gene of said nitrile hydratase, a transformant containing said recombinant plasmid, a method of using said transformant for producing said enzyme, and a method of using said transformant for producing the corresponding amide compound from a nitrile compound.

The present invention provides the amino acid sequence and base sequence of a <u>Pseudonocardia thermophila</u>-derived nitrile hydratase, provides further a method for changing its amino acid sequence and base sequence without substantially changing the functions of said nitrile hydratase, and nitrile hydratases having a base sequence and an amino acid sequence as changed on the basis of said method, and provides furthermore a recombinant plasmid having the gene of said nitrile hydratase, a transformant containing said recombinant plasmid, a method of using said transformant for producing said enzyme, and a method of using said transformant for producing the corresponding amide compound from a nitrile compound.

SEQUENCE LISTING

	(1) SEQ	ID NO	:1:											
5	Sequen	ce Len	gth: 3	205										
	Sequen	се Тур	e: am:	ino a	cid									
10	Topolo	gy: li	near											
,0	Molecu	le Typ	e: pro	otein	1									
	Origin	al Sou	rce:											
15	Orga	nism N	ame:	Pseud	lono	card	<u>lia</u>	the	rmo	phi]	<u>la</u>			
	Stra	in Nam	e: JC1	13095	5									
	Immedi	ate So	urce:											
20	Clon	e Name	: pPT	-DBl						•				
	-	ce Fea												
25		tifica												
		r Info			nitr	ile	hyd	lrat	ase	α	subu	ınit		•
	Sequen	ce Des	cript	ion:										
30	Net T	hr Glu	Asn Il	e Leu	Дrg	Lys	Ser	λsp	Glu	Glu	lle	Gln	Lys	Glu
		•		5				10					15	
35	lle T	hr Ala		l Lys	Лla	Leu		Ser	Net	Leu	lle		Gin	Gly
			20				25					30		
	lle l	eu Thr	Thr Se	r Het	lle		Arg	Net	Ala	Glu		Tyr	Glju	λsπ
40	61 1	35			61	40	•		W - 1	w	45	43-	.	7 L
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50	L) S	Jio Leu		5	u.,		01	90	0.0	лор			95	
	Glo i	Asn Thr			Hic	1[i s	Val		Ya 1	Cvs	Thr	Leu		Ser
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			100				103					110		

	Cys Tyr	Pro Trp	Pro Va	i Leu	Gly	Leu	Pro	Pro	Asn	Trp	Phe	Lys	Glu
		115			120					125			
5	Pro Gla	Tyr Arg	Ser Ar	g Yal	Ya 1	λrg	Glu	Pro	Arg	G 1 n	Lcu	Lcu	Lys
	130	1	•	135					140				
	Glu Glu	Phe Gly	Phe G1	u Yal	Pro	Pro	Ser	Lys	Glu	l 1 e	Lys	Val	Trp
,,,	145		15	0				155					160
	. Asp Ser	Ser Ser	Glu Ne	t Arg	Phe	Yal	Y a i	Leu	Pro	Gln	Årg	Pro	Ala
15			165				170					175	
	Gly Thi	r Asp Gly	Trp Se	r Glu	Glu	Glu	Leu	Ala	Thr	Leu	Ya 1	Thr	Arg
		180)			185					190		
20	Glu Se	r Net Ile	e Gly Ya	1 Glu	Pro	Ala	Lys	Ala	Val	Лla			
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25												•	
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	_	nce Des										_	
	Net As	n Gly Va	l Tyr A	sp Yal	Gly	Gly	Thr	λsp	61y	leu	Gly		
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	Asn	Arg	Pro	Ala	Asp	Glu	Pro	Yal	Phe	Åгg	Ala	Glu	Trp	C 1 u	Lys	Yal
				20					25					30		
5	Ala	Phe	Ala	Net	Phe	Pro	Ala	Thr	Phc	Arg	Ala	Gly	Phe	Net	Gly	Leu
			35					40					45			
10	Asp	G 1 u	Phe	Arg	Phe	Gly	lle	Glu	Gln	¥e t	Asn	Pro	Ala	Glu	Tyr	Lcu
		50					55					60				
	Glu	Ser	Рго	Туг	Tyr	Trp	ll i s	Trp	lle	Arg	Thr	Tyr	Пe	llis	llis	Gly
15	65					70					75					80
	Val	Arg	Thr	Gly	Lys	lle	Аsр	Leu	C l u	Glu	Leu	G 1 u	Årg	Årg	Thr	Gln
			•		85					90					95	
20	Ťyr	Tyr	Arg	Glu	Asn	Pro	λsp	Дlа	Pro	Leu	Pro	Glu	llis	Glu	Gln	Lys
				100					105					110		
25	Pro	Glu	Leu	lle	Glu	Phe	Yal	Asn	Gln	Ála	Val	Tyr	Gly	Gly	Leu	Pro
25			115					120					125			
	λla	Ser	Arg	Glu	V a 1	Аsр	λrg	Pro	Pro	Lys	Phe	Lys	Glu	G 1 y	Asp	Yal
30		130					135					140				
•	Val	Årg	Phe	Ser	Ťhr	Ala	Ser	Pro	Lys	Gly	His	Ala	Arg	Årg	Ala	λrg
	145					150					155					160
35	Tyr	Val	Arg	Gly	Lys	Thr	Gly	Thr	V a l	Y a l	Lys	His	His	Gly	Ala	Tyr
					165					170					175	
40	lle	Tyr	Pro	Asp	Thr	Аlа	Gly	Asn	Gly	Leu	Gly	Glu	Cys	Pro	Glu	llis
				180					185					190		•
	Leu	Tyr	Thr	V a l	Arg	Phe	Thr	Ala	Gln	Glu	Leu	Trp	Gly	Pro	Glu	Gly
45			195					200					205			
	Asp	Pro	λsπ	Ser	Ser	V a 1	Tyr	Tyr	Åsр	Cys	Trp	Glu	Pro	Tyr	i 1 e	Ç 1 u
		210					215					220				
50	Leu	V a 1	Хsр	Thr	Lys	Аlа	Ala	Ala	Аlа							
	225					230			233							

(3) SEQ ID NO:3:

Sequence Length: 702

	Sequence Length: 618	
5	Sequence Type: nucleic acid	
	Strandedness: double	
10	Topology: linear	
	Molecule Type: genomic DNA	
	Original Source:	
15	Organism Name: Pseudonocardia thermophila	
	Strin Name: JCM3095	
	Immediate Source:	
20	Clone Name: pPT-DBl	
	Sequence Feature:	
2 5	Identification Method: E	
	Other Information: nitrile hydratase $lpha$ subunit	
	Sequence Description:	
30	ATGACCGAGA ACATCCTGCG CAAGTCGGAC GAGGAGATCC AGAAGGAGAT CACGGCGCGG	60
	GTCAAGGCCC TGGAGTCGAT GCTCATCGAA CAGGGCATCC TCACCACGTC GATGATCGAC	120
35	CGGATGGCCG AGATCTACGA GAACGAGGTC GGCCCGCACC TCGGCGCGAA GGTCGTCGTG	180
	AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC	240
	AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC	300
40	GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG	360
-	CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG	420
4.5	CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG	480
45	GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG	540
	TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG	600
50	GCGAAGGCGG TCGCGTGA	618
	(4) SEQ ID NO:4:	

Sequence Type: nucleic acid

_	Strandedness: double	
5	Topology: linear	•
•	Molecule Type: genomic DNA	
10	Original Source:	
	Organism Name: Pseudonocardia thermophila	
	Strain Name: JCM3095	
15	Immediate Source:	
	Clone Name: pPT-DB1	
20	Sequence Feature:	
20	Identification Method: E	
	Other Information: nitrile hydratase eta subunit	
25	Sequence Description:	
•	ATGAACGGCG TGTACGACGT CGGCGGCACC GATGGGCTGG GCCCGATCAA CCGGCCCGCG	60
	GACGAACCGG TCTTCCGCGC CGAGTGGGAG AAGGTCGCGT TCGCGATGTT CCCGGCGACG	120
30	TTCCGGGCCG GCTTCATGGG CCTGGACGAG TTCCGGTTCG GCATCGAGCA GATGAACCCG	180
	GCCGAGTACC TCGAGTCGCC GTACTACTGG CACTGGATCC GCACCTACAT CCACCACGGC	240
35	GTCCGCACCG GCAAGATCGA TCTCGAGGAG CTGGAGCGCC GCACGCAGTA CTACCGGGAG	300
	AACCCCGACG CCCCGCTGCC CGAGCACGAG CAGAAGCCGG AGTTGATCGA GTTCGTCAAC	360
	CAGGCCGTCT ACGGCGGGCT GCCCGCAAGC CGGGAGGTCG ACCGACCGCC CAAGTTCAAG	420
40	GAGGGCGACG TGGTGCGGTT CTCCACCGCG AGCCCGAAGG GCCACGCCCG GCGCGCGCG	480
	TACGTGCGCG GCAAGACCGG GACGGTGGTC AAGCACCACG GCGCGTACAT CTACCCGGAC	540
	ACCGCCGGCA ACGGCCTGGG CGAGTGCCCC GAGCACCTCT ACACCGTCCG CTTCACGGCC	600
45	CAGGAGCTGT GGGGGCCGGA AGGGGACCCG AACTCCAGCG TCTACTACGA CTGCTGGGAG	660
	CCCTACATCG AGCTCGTCGA CACGAAGGCG GCCGCGGCAT GA	702
5 0		
	(5) SEQ ID NO:5:	
	Sequence Length: 20	
5 5	Sequence Type: nucleic acid	

	octanacament beinger	
5	Topology: linear	
•	Sequence Type: other nucleic acid; synthetic DNA	
	Sequence Feature: N is A, C, G or T.	
10	Sequence Description:	
	ACNGARAAYA TNYTNMGNAA	20
15	(6) SEQ ID NO:6:	
	Sequence Length: 20	
	Sequence Type: nucleic acid	
20	Strandedness: single	
	Topology: linear	
25	Molecule Type: other nucleic aicd; synthetic DNA	
	Sequence Feature: N is A, C, G or T.	
30	Sequence Description:	
30	TTNCKNARNA TRTTYTCNGT	20
	(7) SEQ ID NO:7:	
35	Sequence Length: 20	
	Sequence Type: nucleic acid	
40	Strandedness: single	
	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
45	Sequence Feature: N is A, C, G or T.	
	Sequence Description:	
50 _	ATGAAYGGNG TNTAYGANGT	20
	(8) SEQ ID NO:8:	
	Common Longths 20	

5	Sequence Type: nucleic acid	
	Strandedness: single	
	Topology: linear	
10	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Feature: N is A, C, G or T.	
15	Sequence Description:	
	ACNTCRTANA CNCCRTTCAT	20
20		
20	(9) SEQ ID NO:9:	
	Sequence Length: 18	
25	Sequence Type: nucleic acid	
	Strandedness: single	
30	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
35	AACATCATGC GCAAGTCG	18
40	(10) SEQ ID NO:10:	
	Sequence Length: 17	
	Sequence Type: nucleic acid	
45	Strandedness: single	
	Topology: linear	
50	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	CAGGAAACAG CTATGAC	17
55	<u>.</u>	

	(II) SEQ ID NO.II.	
	Sequence Length: 20	
5	Sequence Type: nucleic acid	
	Strandedness: single	•
10	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
15	GGCCAGTGCC TAGCTTACAT	20
20	(12) SEQ ID NO:12:	
	Sequence Length: 17	
25	Sequence Type: nucleic acid	
25	Strandedness: single	
	Topology: linear	
30	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	GTTTTCCCAG TCACGAC	17
35		
	(13) SEQ ID NO:13:	
40	Sequence Length: 18	
	Sequence Type: nucleic acid	
	Strandedness: single	
45	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
50	Sequence Description:	
	AACATCACGC GCAAGTCG	18
55		
	(14) SEO ID NO.14	

	Sequence Type: nucleic acid	
5	Strandedness: single	
	Topology: linear	
10	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	AACATCGCGC GCAAGTCG	18
15		
	(15) SEQ ID NO:15	
•	Sequence Length: 18	
20	Sequence Type: nucleic acid	
	Strandedness: single	
25	Topology: linear	
	Molecule Type; other nucleic acid; synthetic DNA	
	Sequence Description:	
30	AACATCGTGC GCAAGTCG	18
35	(16) SEQ ID NO:16:	
	Sequence Length: 18	
	Sequence Type: nucleic acid	
40	Strandedness: single	
	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
45	Sequence Description:	
	ATCACGGTGC GGGTCAAG	18
50		
	(17) SEQ ID NO:17:	
	Sequence Length: 18	
5 5 ·	Sequence Type: nucleic acid	

	Strandedness: single	
5	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
10	ACGTCGTTGA TCGACCGG	18
	,	
	(18) SEQ ID NO:18:	
15	Sequence Length: 18	
	Sequence Type: nucleic acid	
20	Strandedness: single	
20	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
25	Sequence Description:	
		18
	GACGGCTCCG AGGCCTGC	
	GACGGCTCCG AGGCCTGC	
30	GACGGCTCCG AGGCCTGC (19) SEQ ID NO:19:	
30		
30	(19) SEQ ID NO:19:	
	(19) SEQ ID NO:19: Sequence Length: 18	
	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid	
	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single	
35	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single Topology: linear	
<i>35</i>	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single Topology: linear Molecule Type: other nucleic acid; synthetic DNA	18
35	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single Topology: linear Molecule Type: other nucleic acid; synthetic DNA Sequence Description:	18
<i>35</i>	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single Topology: linear Molecule Type: other nucleic acid; synthetic DNA Sequence Description:	18
<i>35</i>	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single Topology: linear Molecule Type: other nucleic acid; synthetic DNA Sequence Description: CTGCAGGCCG AGGACATG	18
35 40 45	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single Topology: linear Molecule Type: other nucleic acid; synthetic DNA Sequence Description: CTGCAGGCCG AGGACATG (20) SEQ ID NO:20:	18
35 40 45	(19) SEQ ID NO:19: Sequence Length: 18 Sequence Type: nucleic acid Strandedness: single Topology: linear Molecule Type: other nucleic acid; synthetic DNA Sequence Description: CTGCAGGCCG AGGACATG (20) SEQ ID NO:20: Sequence Length: 18	18

	Molecule Type: other nucleic acid; synthetic DNA	
5	Sequence Description:	
-	GACGAGGCCC ACCACGTC	18
	(21) SEQ ID NO:21:	
10	Sequence Length: 18	
	Sequence Type: Nucleic acid	
15	Strandedness: single	
,,	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
20	Sequence Description:	
	CACGTCATCG TGTGCACG	18
25	(22) SEQ ID NO:22:	
	Sequence Length: 18	
30	Sequence Type: nucleic acid	
	Strandedness: single	
	Topology: linear	
35	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	AACTGGTACA AGGAGCCG	18
40	(23) SEQ ID NO:23:	
	Sequence Length: 18	
45	Sequence Type: nucleic acid	
	Strandedness: single	
	Topology: línear	
50	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	

	GAGCCGGAGT ACCGCTCC	18
5	(24) SEQ ID NO:24:	
	Sequence Length: 18	
10	Sequence Type: nucleic acid	
	Strandedness: single	•
	Topology: linear	
15	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	CGGCAGGTGC TCAAGGAG	18
	(25) SEQ ID NO:25:	
	Sequence Length: 18	
25	Sequence Type: nucleic acid	
	Strandedness: single	
30	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
35	AAGGAGGACT TCGGCTTC	18
	(26) SEQ ID NO:26:	
40	Sequence Length: 18	
	Sequence Type: nucleic acid	
•	Strandedness: single	•
45	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	*
50	Sequence Description:	
	GAGCTCACCA CCCTCGTC	18

	(27) BEQ 15 NO.27.	
5	Sequence Length: 18	
	Sequence Type: nucleic acid	
	Strandedness: single	
10	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
15	-CGCGAGTTGA TGATCGGC	18
	(28) SEQ ID NO:28:	
20	Sequence Length: 18	
	Sequence Type: nucleic acid	
25	Strandedness: single	
	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
30	Sequence Description:	
	GCGAAGGAGG TCGCGTGA	18
35	(29) SEQ ID NO:29:	
	Sequence Length: 18	
	Sequence Type: nucleic acid	
40	Strandedness: single	
	Topology: linear	
45	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	CGGCCCGTGG ACGAACCG	18
50	(20) SEQ ID NO:30:	٠
	Sequence Length: 18	

	Sequence Type: nucleic acid	
5	Strandedness: single	
	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
10	Sequence Description:	
	CCCGCGAACG AACCGGTC	18
15	(31) SEQ ID NO:31:	
	Sequence Length: 18	
20	Sequence Type: nucleic acid	
20	Strandedness: single	
÷	Topology: linear	
25	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
30	CTGCCCGATC ACGAGCAG	18
	(32) SEQ ID NO:32:	
	Sequence Length: 18	
35	Sequence Type: nucleic acid	
	Strandedness: single	
40	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
45	CTGCCCCGC ACGAGCAG	18
	(33) SEQ ID NO:33:	٠
50	Sequence Length: 18	
	Sequence Type: nucleic acid	
	Strandedness: single	

	Topology: linear	
5	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	CTGCCCTCGC ACGAGCAG	18
10 '	(24) GEO TE NO.24.	
	(34) SEQ ID NO:34:	*
	Sequence Length: 18	
15	Sequence Type: nucleic acid	
	Strandedness: single	
	Topology: linear	
20	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
25	CTGCCCGGC ACGAGCAG	18
	(35) SEQ ID NO:35:	
30	Sequence Length: 18	
	Sequence Type: nucleic acid	
	Strandedness: single	
35	Topology: linear	•
`	Molecule Type: other nucleic acid; synthetic DNA	
•	Sequence Description:	
40	CTGCCCTGCC ACGAGCAG	18
,	, (25), 670, 77, Wallet	
45	(36) SEQ ID NO:36:	
***	Sequence Length: 18	
	Sequence Type: nucleic acid	
50	Strandedness: single	
	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
55		

	Sequence Description.	
5	CTGCCCTGC ACGAGCAG	18
	(37) SEQ ID NO:37:	
10	Sequence Length: 18	
	Sequence Type: nucleic acid	
	Strandedness: single	
15	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
20	Sequence Description:	
20	CTGCCCACGC ACGAGCAG	18
	(38) SEQ ID NO:38:	
25	Sequence Length: 18	
	Sequence Type: nucleic acid	
30	Strandedness: single	
	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
35	Sequence Description:	
	TTCACGGACC AGGAGCTG	18
40	(39) SEQ ID NO:39:	
	Sequence Length: 18	
	Sequence Type: nucleic acid	
45	Strandedness: single	
	Topology: linear	
50	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	TTCACGATCC AGGAGCTG	18
55		

	(40) SEQ ID NO.40.	
5	Sequence Length: 18	
	Sequence Type: nucleic acid	
	Strandedness: single	
10	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
15	Sequence Description:	
	TTCACGGTCC AGGAGCTG	18
20	(41) SEQ ID NO:41:	
	Sequence Length: 18	
	Sequence Type: nucleic acid	
25	Strandedness: single	*
•	Topology: linear	
	Molecule Type: other nucleic acid; synthetic DNA	
30	Sequence Description:	
	TTCACGGAGC AGGAGCTG	18
35	(42) SEQ ID NO:42:	
	Sequence Length: 18	
40	Sequence Type: nucleic acid	
	Strandedness: single	
	Topology: linear	
45 .	Molecule Type: other nucleic acid; synthetic DNA	
	Sequence Description:	
	CCGAACTACA GCGTCTAC	18
5 0		

5

SEQUENCE LISTING

5	(1) GENE	RAL INFORMATION:
	(i)	APPLICANT: Mitsui Toatsu Chemicals, Inc.
10	(ii)	TITLE OF INVENTION: NITRILE HYDRATASE
	(iii)	NUMBER OF SEQUENCES: 42
15	(iv)	CORRESPONDENCE ADDRESS: (A) ADDRESSEE: GRAHAM WATT & CO. (B) STREET: Riverhead (C) CITY: Sevenoaks (D) COUNTY: Kent (E) COUNTRY: United Kingdom (F) POSTAL CODE: TN13 2BN
25	(V)	COMPUTER READABLE FORM: (A) MEDIUM TYPE: Floppy disk (B) COMPUTER: IBM PC compatible (C) OPERATING SYSTEM: PC-DOS/MS-DOS (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
30	(vi)	CURRENT APPLICATION DATA: (A) APPLICATION NUMBER: EP 97300932.7 (B) FILING DATE: 13-FEB-1997
35	(vii)	PRIOR APPLICATION DATA: (A) APPLICATION NUMBER: JP 8-027004 (B) FILING DATE: 14-FEB-1996
	(viii)	ATTORNEY/AGENT INFORMATION: (A) NAME: HOLDCROFT, Dr. James Gerald (B) REFERENCE/DOCKET NUMBER: 12288
40	(ix)	TELECOMMUNICATION INFORMATION: (A) TELEPHONE: (1732) 450055 (B) TELEFAX: (1732) 450113
45	(2) INFOR	RMATION FOR SEQ ID NO:1:
50	(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 205 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear

62 -

	(ii)	MOL	ECUL	E TY	PE:	prot	ein					٠				
5	(vi)	(A) OR	L SO GANI: RAIN	SM:	Pseu		card	ia t	herm	ophi	la				
10	(vii)			te so One:			•				٠					
	(ix)	(A (B) NA	me/k) Cati	ON:	12	05	/no	te=	" Ide	ntif.	icat	ion :	Meth	od: 1	e "
15	(ix)	(A (B) NAI	ME/KI CATIO	ON:	12	05	/ m a i	•	g		L				
20	subuni	t"	, 01	nek .	INFO		ION:	/ no	ce≖	"nit:	riie	hyd:	rata	se a	lpha	
	(xi)	SEQ	UENC	e de:	SCRI	PTIO	N: S	EQ II	D NO	:1:						
25	Met 1	Thr	Glu	Asn	Ile 5	Leu	Arg	Lys	Ser	Asp 10	Glu	Glu	Ile	Gln	Lys 15	Glu
	Ile	Thr	Ala	Arg 20	Val	Lys	Ala	Leu	Glu 25	Ser	Met	Leu	Ile	Glu 30	Gln	Gly
30	Ile	Leu	Thr 35	Thr	Ser	Met	Ile	Asp 40	Arg	Met	Ala	Glu	Ile 45	Tyr	Glu	Asn
	Glu	Val 50	Gly	Pro	His	Leu	Gly 55	Ala	Lys	Val	Val	Val 60	Lys	Ala	Trp	Thr
35	Asp 65	Pro	Glu	Phe	Lys	Lys 70	Arg	Leu	Leu	Ala	Asp 75	Gly	Thr	Glu	Ala	Cys 80
40	Lys	Glu	Leu	Gly	Ile 85	Gly	Gly	Leu	Gln	Gly 90	Glu	Asp	Met	Met	Trp 95	Val
70	Glu	Asn	Thr	Asp 100	Glu	Val	His	His	Val 105	Val	Val	Cys	Thr	Leu 110	Cys	Ser
45	Суз	Tyr	Pro 115	Trp	Pro	Val	Leu	Gly 120	Leu	Pro	Pro	Asn	Trp 125	Phe	Lys	Glu
	Pro	Gln 130	Tyr	Arg	Ser	Arg	Val 135	Val	Arg	Glu	Pro	Arg 140	Gln	Leu	Leu	Lys
50																

	, Glu 145	Glu	Phe	Gly	Phe	Glu 150	Val	Pro	Pro	Ser	Lys 155	Glu	Ile	Lys	Val	Trp 160
5	Asp	Ser	Ser	Ser	Glu 165	Met	Arg	Phe	Val	Val 170	Leu	Pro	Gln	Arg	Pro 175	Ala
10	Gly	Thr	Asp	Gly 180	Trp	Ser	Glu	Glu	Glu 185	Leu	Ala	Thr	Leu	Val 190	Thr	Arg
	Glu	Ser	Met 195	Ile	Gly	Val	Glu	Pro 200	Ala	Lys	Ala	Val	Ala 205			
15	(2) INFO	RMAT:	ION I	FOR S	SEQ :	ID NO	0:2:					,				
20	(i)	(A (B) (C)	JENCI) LEI) TYI) STI) TOI	NGTH: PE: 4 RANDI	23: mino ZDNES	am: cac:	ino a id sing:	acid	3 .							
	(ii)	MOL	CULE	TYI	PE: 1	prote	ein									
25	(vi)	(A)	GINAI ORC STI	GANIS	5M: 1	euc		card	la th	nermo	phi]	l a				
30	(vii)		EDIAT													
	(ix)	(A)	TURE: NAM LOC OTH	Æ/KI Catio	ON: 1	t23	33	/not	e= '	'Ider	ıtifi	cati	ion 1	letho	od:	E"
35	(ix)	(A) (B)	NAM LOC	Æ/KE CATIO	N: 1	123	33	/not				المراجعة المراجعة	-		. 4.	
40	subunit	t"	OTE	101()	in or	W. T. J	.on.	/1101	.e-	nici	TIE	пуат	acas	se De	eta .	
	(xi)	SEQU	JENCE	e des	CRIE	PTION	l: SE	11 Q	NO:	2:				٠		
45	Met 1	Asn	Gly	Val	Tyr 5	Asp	Val	Gly	Gly	Thr 10	Asp	Gly	Leu	Gly	Pro 15	Ile
•	Asn	Arg	Pro	Ala 20	Aap	Glu	Pro	Val	Phe 25	Arg	Ala	Glu	Trp	Glu 30	Lys	Val
50																,

	Ala	Phe	Ala 35	Met	Phe	Pro	Ala	Thr 40	Phe	Arg	Ala	Gly	Phe 45	Met	Gly	Leu
5	Asp	Glu 50	Phe	Arg	Phe	Gly	Ile 55	Glu	Gln	Met	Asn	Pro 60	Ala	Glu	Tyr	Leu
10	Glu 65	Ser	Pro	Tyr	Tyr	Trp 70	His	Trp	Ile	Arg	Thr 75	Tyr	Ile	His	His	Gly 80
	Val	Arg	Thr	Gly	Lys 85	Ile	Asp	Leu	Glu	Glu 90	Leu	Glu	Arg	Arg	Thr 95	Gln
15	Tyr	Tyr	Arg	Glu 100	Asn	Pro	Asp	Ala	Pro 105	Leu	Pro	Glu	His	Glu 110	Gln	Lys
	Pro	Glu	Leu 115	Ile	Glu	Phe	Val	Asn 120	Gln	Ala	Val	Tyr	Gly 125	Gly	Leu	Pro
20	Ala	Ser 130	Arg	Glu	Val	Asp	Arg 135	Pro	Pro	Lys	Phe	Lys 140	Glu	Gly	Asp	Val
	Val 145	Arg	Phe	Ser	Thr	Ala 150	Ser	Pro	Lys	Gly	His 155	Ala	Arg	Arg	Ala	Arg 160
25	Tyr	Val	Arg	Gly	Lys 165	Thr	Gly	Thr	Val	Val 170	Lys	His	His	Gly	Ala 175	Tyr
	Ile	Tyr	Pro	Asp 180	Thr	Ala	Gly	Asn	Gly 185	Leu	Gly	Glu	Cys	Pro 190	Glu	His
30	Leu	Tyr	Thr 195	Val	Arg	Phe	Thr	Ala 200	Gln	Glu	Leu	Trp	Gly 205	Pro	Glu	Gly
35	Asp	Pro 210	Asn	Ser	Ser	Val	Tyr 215	Tyr	Asp	Сув	Trp	Glu 220	Pro	Tyr	Ile	Glu
	Leu 225	Val	Asp	Thr	Lys	Ala 230	Ala	Ala	Ala							
			•							ı						

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 618 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

55

45

CGGATGCCG AGATCTACGA GAACGAGGTC GGCCCGCACC TCGGCGCGAA GGTCGTCGTG AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		(vi) ORIGINAL SOURCE:(A) ORGANISM: Pseudonocardia thermophila(B) STRAIN: JCM3095	
(A) NAME/REY: misc_feature (B) LOCATION: 1618 (D) OTHER INFORMATION: /note= "Identification Method: E" (ix) FEATURE: (A) NAME/REY: misc_feature (B) LOCATION: 1618 (C) OTHER INFORMATION: /note= "nitrile hydratase alpha subunit" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3: ATGACCGAGA ACATCCTGCG CAAGTCGGAC GAGGAGATCC AGAAGGAGAT CACGGCGCGG GTCAAGGCCC TGGAGTCGAT GCTCATCGAA CAGGGCATCC TCACCACGTC GATGATCGAC CGGATGGCCC AGATCTACGA GAACGAGGCT GGCCCGCAC TCGGCGCGAA GGTCGTCGTG AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCG GCTCGAGGG GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGC GGTGCTGGGG CAGGCCCGGG CAGGCCCGG CAGGCCCGG CAGGCCCGG CAGGCCCGG CAGGCCCGG CAGGCCCCGG CAGGCCCCGG CAGGCCCGG CAGGCCCGG CAGGCCCGG GAGCCCCGGG CACCGACGGG TGGAGCCCGG CACCCACGG CACCCCCGGG CACCCACGGG CACCGACGGG TGGAGCCCGG CACCCACGG CACCCACGGG CACCCACGGG CACCGACGGG CACCCACC	5		
(A) NAME/KEY: misc feature (B) LOCATION: 1618 (D) OTHER INFORMATION: /note= "nitrile hydratase alpha subunit" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3: ATGACCGAGA ACATCCTGCG CAAGTCGGAC GAGGAGATCC AGAAGGAGAT CACGGCGCGG GTCAAGGCCC TGGAGTCGAT GCTCATCGAA CAGGGCATCC TCACCACGTC GATGATCGAC CGGATGGCCG AGATCTACGA GAACGAGGTC GGCCCGCACC TCGGCGCGAA GGTCGTCGTG AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCC CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCCAG GCCCCGCGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	10	(A) NAME/KEY: misc_feature (B) LOCATION: 1618	
ATGACCGAGA ACATCCTGCG CAAGTCGGAC GAGGAGATCC AGAAGGAGAT CACGGCGCGG GTCAAGGCCC TGGAGTCGAT GCTCATCGAA CAGGGCATCC TCACCACGTC GATGATCGAC CGGATGGCCG AGATCTACGA GAACGAGGTC GGCCCGCACC TCGGCGCGAA GGTCGTCGTG AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	15	(A) NAME/KEY: misc_feature (B) LOCATION: 1618 (D) OTHER INFORMATION: /note= "nitrile hydratase alpha	
GTCAAGGCCC TGGAGTCGAT GCTCATCGAA CAGGGCATCC TCACCACGTC GATGATCGAC CGGATGGCCG AGATCTACGA GAACGAGGTC GGCCCGCACC TCGGCGCGAA GGTCGTCGTG AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:	
25 AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGCAC CGAGGCCTGC AAGGAGCTCG CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	20	ATGACCGAGA ACATCCTGCG CAAGTCGGAC GAGGAGATCC AGAAGGAGAT CACGGCGCGG	60
AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		GTCAAGGCCC TGGAGTCGAT GCTCATCGAA CAGGGCATCC TCACCACGTC GATGATCGAC	120
AAGGCCTGGA CCGACCCGGA GFTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		CGGATGGCCG AGATCTACGA GAACGAGGTC GGCCCGCACC TCGGCGCGAA GGTCGTCGTG	180
GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	25	AAGGCCTGGA CCGACCCGGA GTTCAAGAAG CGTCTGCTCG CCGACGGCAC CGAGGCCTGC	240
CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		AAGGAGCTCG GCATCGGCGG CCTGCAGGGC GAGGACATGA TGTGGGTGGA GAACACCGAC	300
CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	20	GAGGTCCACC ACGTCGTCGT GTGCACGCTC TGCTCCTGCT ACCCGTGGCC GGTGCTGGGG	360
GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGG CACCGACGGG TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	30	CTGCCGCCGA ACTGGTTCAA GGAGCCGCAG TACCGCTCCC GCGTGGTGCG TGAGCCCCGG	420
TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		CAGCTGCTCA AGGAGGAGTT CGGCTTCGAG GTCCCGCCGA GCAAGGAGAT CAAGGTCTGG	480
GCGAAGGCGG TCGCGTGA (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	35	GACTCCAGCT CCGAGATGCG CTTCGTCGTC CTCCCGCAGC GCCCCGCGGG CACCGACGGG	540
(2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		TGGAGCGAGG AGGAGCTCGC CACCCTCGTC ACCCGCGAGT CGATGATCGG CGTCGAACCG	600
(2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 702 base pairs (B) TYPE: nucleic acid		GCGAAGGCGG TCGCGTGA	618
(A) LENGTH: 702 base pairs (B) TYPE: nucleic acid	10	(2) INFORMATION FOR SEQ ID NO:4:	
, ,	15	(A) LENGTH: 702 base pairs	

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(D) TOPOLOGY: linear

5	(ii) MOLECULE TYPE: DNA (genomic)	
	(vi) ORIGINAL SOURCE:(A) ORGANISM: Pseudonocardia thermophila(B) STRAIN: JCM3095	
10	(vii) IMMEDIATE SOURCE: (B) CLONE: pPT-DB1	
15	<pre>(ix) FEATURE: (A) NAME/KEY: misc_feature (B) LOCATION: 1702 (D) OTHER INFORMATION: /note= "Identification Method: E."</pre>	
	<pre>(ix) FEATURE: (A) NAME/KEY: misc_feature (B) LOCATION: 1702</pre>	
20	(D) OTHER INFORMATION: /note= "nitrile hydratase beta subunit"	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
25	ATGAACGGCG TGTACGACGT CGGCGGCACC GATGGGCTGG GCCCGATCAA CCGGCCCGCG	60
	GACGAACCGG TCTTCCGCGC CGAGTGGGAG AAGGTCGCGT TCGCGATGTT CCCGGCGACG	120
	TTCCGGGCCG GCTTCATGGG CCTGGACGAG TTCCGGTTCG GCATCGAGCA GATGAACCCG	180
30	GCCGAGTACC TCGAGTCGCC GTACTACTGG CACTGGATCC GCACCTACAT CCACCACGGC	240
	GTCCGCACCG GCAAGATCGA TCTCGAGGAG CTGGAGCGCC GCACGCAGTA CTACCGGGAG	300
	AACCCCGACG CCCCGCTGCC CGAGCACGAG CAGAAGCCGG AGTTGATCGA GTTCGTCAAC	360
35	CAGGCCGTCT ACGGCGGGCT GCCCGCAAGC CGGGAGGTCG ACCGACCGCC CAAGTTCAAG	420
	GAGGGCGACG TGGTGCGGTT CTCCACCGCG AGCCCGAAGG GCCACGCCCG GCGCGCGCG	480
	TACGTGCGCG GCAAGACCGG GACGGTGGTC AAGCACCACG GCGCGTACAT CTACCCGGAC	540
40	ACCGCCGGCA ACGGCCTGGG CGAGTGCCCC GAGCACCTCT ACACCGTCCG CTTCACGGCC	600
	CAGGAGCTGT GGGGGCCGGA AGGGGACCCG AACTCCAGCG TCTACTACGA CTGCTGGGAG	660
45	CCCTACATCG AGCTCGTCGA CACGAAGGCG GCCGCGGCAT GA	702

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	(2) INFORMATION FOR SEQ ID NO:5;	
5	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
10	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
15	<pre>(ix) FEATURE: (A) NAME/KEY: misc_feature (B) LOCATION: 318 (D) OTHER INFORMATION: /note= "Nucleotides 3, 12, 15, and 18 wherein N = A, C, G, or T."</pre>	
·	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:	
20	ACNGARAAYA TNYTNMGNAA	20
	(2) INFORMATION FOR SEQ ID NO:6:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
30	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	•
35	<pre>(ix) FEATURE: (A) NAME/KEY: misc_feature (B) LOCATION: 318 (D) OTHER INFORMATION: /note= "Nucleotides 3, 6, 9, and 18 wherein N = A, C, G, or T."</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:	
	TTNCKNARNA TRTTYTCNGT	20
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	(2) INFORMATION FOR SEQ ID NO:7:	
45	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid	

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	(C) STRANDEDNESS: single (D) TOPOLOGY: linear	
5	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
10	<pre>(ix) FEATURE: (A) NAME/KEY: misc_feature (B) LOCATION: 918 (D) OTHER INFORMATION: /note= "Nucleotides 9, 12, and 18 wherein N = A, C, G, or T."</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:	
15	ATGAAYGGNG TNTAYGANGT	20
	(2) INFORMATION FOR SEQ ID NO:8:	
20	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
25	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
30	<pre>(ix) FEATURE: (A) NAME/KEY: misc_feature (B) LOCATION: 312 (D) OTHER INFORMATION: /note= "Nucleotides 3, 9, and 12 wherein N = A, C, G, or T."</pre>	-
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:	
35	ACNTCRTANA CNCCRTTCAT	20
	(2) INFORMATION FOR SEQ ID NO:9:	
40	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
45	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	

AACATCATGC GCAAGTCG (2) INFORMATION FOR SEQ ID NO:10: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 17 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	18
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 17 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(A) LENGTH: 17 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	•
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:	
CAGGAAACAG CTATGAC	17
(2) INFORMATION FOR SEQ ID NO:11:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	.*
<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:	
GGCCAGTGCC TAGCTTACAT	20
35 (2) INFORMATION FOR SEQ ID NO:12:	,
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 17 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	ŕ
<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
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	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:	
5	GTTTTCCCAG TCACGAC	17
	(2) INFORMATION FOR SEQ ID NO:13:	
1 0	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:	
	AACATCACGC GCAAGTCG	18
20	(2) INFORMATION FOR SEQ ID NO:14:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	
30	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:	
	AACATCGCGC GCAAGTCG	18
35	(2) INFORMATION FOR SEQ ID NO:15:	
	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid	
40	<pre>(C) STRANDEDNESS: single (D) TOPOLOGY: linear</pre>	
	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
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	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:	
5	AACATCGTGC GCAAGTCG	18
	(2) INFORMATION FOR SEQ ID NO:16:	
10	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
20	ATCACGGTGC GGGTCAAG	18
	(2) INFORMATION FOR SEQ ID NO:17:	
25	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
30	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:	
	ACGTCGTTGA TCGACCGG	18
<i>35</i>	(2) INFORMATION FOR SEQ ID NO:18:	
40	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
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	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:	
5	GACGGCTCCG AGGCCTGC	18
	(2) INFORMATION FOR SEQ ID NO:19:	
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	•
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	-
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:	
20	CTGCAGGCCG AGGACATG	18
	(2) INFORMATION FOR SEQ ID NO:20:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	·
30	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:	
35	GACGAGGCCC ACCACGTC	18
	(2) INFORMATION FOR SEQ ID NO:21:	
40	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
45	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	

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	(XI) SEQUENCE DESCRIPTION: SEQ ID NO:21:	
5	CACGTCATCG TGTGCACG	18
	(2) INFORMATION FOR SEQ ID NO:22:	
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	,
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:	
20 ,	AACTGGTACA AGGAGCCG	18
	(2) INFORMATION FOR SEQ ID NO:23:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
30	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:	
	GAGCCGGAGT ACCGCTCC	18
35	(2) INFORMATION FOR SEQ ID NO:24:	
40	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
45	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	

	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:	
5	CGGCAGGTGC TCAAGGAG	18
	(2) INFORMATION FOR SEQ ID NO:25:	
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:	
20	AAGGAGGACT TCGGCTTC	18
	(2) INFORMATION FOR SEQ ID NO:26:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
30	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:	
	GAGCTCACCA CCCTCGTC	18
35	(2) INFORMATION FOR SEQ ID NO:27:	
40	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
45	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	

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	(X1) SEQUENCE DESCRIPTION: SEQ ID NO:27:	
5	CGCGAGTTGA TGATCGGC	18
	(2) INFORMATION FOR SEQ ID NO:28:	
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:	
20	GCGAAGGAGG TCGCGTGA	18
	(2) INFORMATION FOR SEQ ID NO:29:	
25	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
30	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:	
	CGGCCCGTGG ACGAACCG	18
35	(2) INFORMATION FOR SEQ ID NO:30:	
40	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	
45	(A) DEBORIFIZOR, /Wedc - Synchecte Dun	

	(X1) SEQUENCE DESCRIPTION: SEQ ID NO:30:	
5	CCCGCGAACG AACCGGTC	18
	(2) INFORMATION FOR SEQ ID NO:31:	
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:	
20	CTGCCCGATC ACGAGCAG	18
	(2) INFORMATION FOR SEQ ID NO:32:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
30	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:	
	CTGCCCCGC ACGAGCAG	18
35	(2) INFORMATION FOR SEQ ID NO:33:	
40	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
45	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	

	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:	
5	CTGCCCTCGC ACGAGCAG	18
	(2) INFORMATION FOR SEQ ID NO:34:	
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	٠
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:	
	CTGCCCCGGC ACGAGCAG	18
20	(2) INFORMATION FOR SEQ ID NO:35:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
30	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:	
	CTGCCCTGCC ACGAGCAG	18
35	(2) INFORMATION FOR SEQ ID NO:36:	
	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	
40	(D) TOPOLOGY: linear	
	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
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		(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:	
5	CTGC	CCCCTGC ACGAGCAG	18
	(2)	INFORMATION FOR SEQ ID NO:37:	
10		(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
15		(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	
		(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:	
20	CTGC	CCCACGC ACGAGCAG	18
	(2)	INFORMATION FOR SEQ ID NO:38:	
25		(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
30		<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
		(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:	
	TTCA	ACGGACC AGGAGCTG	18
35	(2)	INFORMATION FOR SEQ ID NO:39:	
40		(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
		(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"	
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	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:	
5	TTCACGATCC AGGAGCTG	18
	(2) INFORMATION FOR SEQ ID NO:40:	
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
15	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:	•
20	TTCACGGTCC AGGAGCTG	18
	(2) INFORMATION FOR SEQ ID NO:41:	
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
30	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:	
35	TTCACGGAGC AGGAGCTG	18
	(2) INFORMATION FOR SEQ ID NO:42:	
40	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
45	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "synthetic DNA"</pre>	
5 0	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:	
	CCGAACTACA GCGTCTAC	18
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	Claims	
	A nitrile hydratase which comprises either:	

- (a) one or both of an α -subunit having the amino acid sequence of Sequence Number 1 in Sequence Listing and a β -subunit having the amino acid sequence of Sequence Number 2 in Sequence Listing; or (b) one or both of, as the constitutive element, an α -subunit to be derived from the amino acid sequence of Sequence Number 1 in Sequence Listing through partial substitution, deletion or addition in said sequence, and a β -subunit to be derived from the amino acid sequence of Sequence Number 2 in Sequence Listing through partial substitution, deletion or addition in said sequence.
- The nitrile hydratase according to claim 1 which is derived from Pseudonocardia thermophila.
- 3. A nitrile hydratase according to claim 1 or 2 which comprises, as the constitutive element selected from the group consisting of:
 - (i) an α -subunit to be derived from the amino acid sequence of Sequence Number 1 in Sequence Listing through substitution of one or more amino acids of the 6th, 19th, 36th, 77th, 90th, 102nd, 106th, 126th, 130th, 142nd, 146th, 187th, 194th and 203rd amino acids in said sequence with any other amino acid(s);
 - (ii) a β-subunit to be derived from the amino acid sequence of Sequence Number 2 in Sequence Listing through substitution of one or more amino acids of the 20th, 21st, 108th, 200th and 212th amino acids in said sequence with any other amino acid(s).
- 20 4. A gene which codes for the α-subunit of the nitrile hydratase selected from the group consisting of any one of:
 - (a) claims 1 to 3;

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- (b) having the base sequence of Sequence Number 3 in Sequence Listing;
- (c) having a base sequence to be derived from the base sequence of Sequence Number 3 in Sequence Listing through partial substitution, deletion or addition in said base sequence; or
- (d) having a base sequence to be derived from the base sequence of Sequence Number 3 in Sequence Listing through substitution therein of any one or more base sequence units of from 16th to 18th sequence unit, from 55th to 57th sequence unit, from 112th to 114th sequence unit, from 229th to 231st sequence unit, from 268th to 270th sequence unit, from 304th to 306th sequence unit, from 316th to 318th sequence unit, from 376th to 378th sequence unit, from 388th to 390th sequence unit, from 424th to 426th sequence unit, from 436th to 438th sequence unit, from 559th to 561st sequence unit, from 580th to 582nd sequence unit, and from 607th to 609th sequence unit, with any other base sequence unit(s).
- 5. A gene which codes for the β-subunit of the nitrile hydratase selected from the group consisting of:
- (a) any one of claims 1 to 3;
 - (b) having the base sequence of Sequence Number 4 in Sequence Listing;
 - (c) having a base sequence to be derived from the base sequence of Sequence Number 4 in Sequence Listing through partial substitution, deletion or addition in said base sequence; or
 - (d) having a base sequence to be derived from the base sequence of Sequence Number 4 in Sequence Listing through substitution therein of any one or more base sequence units of from 58th to 60th sequence unit, from 61st to 63rd sequence unit, from 322nd to 324th sequence unit, from 598th to 600th sequence unit, and from 634th to 636th sequence unit, with any other base sequence unit(s).
- 45 6. A recombinant plasmid which has any of the gene coding for the α-subunit of the nitrile hydratase of claim 4, 6 to 10, or the gene coding for the β-subunit of the nitrile hydratase of claim 5.
 - 7. A recombinant plasmid which has the gene coding for the α -subunit of the nitrile hydratase of claim 4, and the gene coding for the β -subunit of the nitrile hydratase of claim 5.
 - 8. A transformant which carries the recombinant plasmid of claim 6 or claim 7.
 - 9. A method for producing a nitrile hydratase, which comprises incubating cells of the transformant of claim 8 followed by collecting the nitrile hydratase formed through the incubation from the cells, the culture, and the product as formed by processing them.
 - 10. A method for producing the corresponding amide compound from a nitrile compound, which comprises incubating cells of the transformant of claim 8 followed by bringing a nitrile compound into contact with the cells, the culture,

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and the product as formed by processing them, or with the nitrile hydratase as obtained from them, in an aqueous medium.

